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CXXX. RESPIRATORY QUOTIENT IN OBESE SUBJECTS.

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Introduction.

A long series of determinations of the B.M.R. in cases of obesity under treatment, accumulated over many months, showed that while the B.M.R. was generally within normal limits, the R.Q. was usually low, and often below 0·70. Quotients below 0·70 are by no means unknown in the work of Benedict on healthy subjects during fasting [*cf.* Benedict and Higgins, 1912]. Lehmann and his co-workers [1893], in the study of a fasting individual, observed low values of the R.Q. which, during several days, averaged 0·67. Bernstein and Falta [1916] obtained average quotients of 0·657 and 0·653 while feeding a normal individual on oatmeal, wheat, and butter; and on two different days quotients as low as 0·631 were recorded. Heinbecker [1931] has reported similarly low values in his study of the metabolism of Eskimos, and it is noteworthy that he specially remarks the relative absence of acetonuria in association with these abnormal values. The very low quotients encountered in diabetes, starvation or after administration of a ketogenic diet are undoubtedly due, in part at least, to ketosis, which means, in effect, the storage of oxygen. This is undoubtedly a factor in the low values reported by Means [1915]. Such an explanation of the results in our non-diabetic obese patients is, however, inadmissible, since the urine of these patients never contained more than a trace of ketones.

There is no doubt, however, that a value for the R.Q. below 0·69 in a diabetic subject, or 0·72 in a normal, is regarded with suspicion as due to faulty technique [*cf.* Lusk, 1928, p. 671], and we therefore considered it essential to institute a rigorous testing of the method by which our low values had been obtained.

Method.

The method used for the determination of the B.M.R. and R.Q. was the open method, using Haldane's face mask and Douglas bags, and analysing the collected expired air with the aid of the Haldane apparatus. This technique was employed simultaneously for the routine hospital work and for other metabolic investigations, in the course of which the very few low values of

the R.Q. found were associated with an unexpectedly low B.M.R., and could well be explained by a leak in the apparatus. In the case, however, of the obese patients on a sub-caloric diet, the very low R.Q. was encountered with a considerable degree of constancy, and was not associated with a reduced metabolic rate.

The points at which technical errors may enter into the determination of the R.Q. are:

(1) the fit of the mask,
(2) leakage from the bags,
(3) faulty analytical technique,
(4) the subject.

(1) *The fit of the mask.* The fitting of the mask was carefully carried out in every case, and tests were always made for leaks before and after the collection of expired air. The subjects co-operated intelligently, themselves pointing out any tendency to leakage between the cushion and the face. The obese person, moreover, has just that type of physiognomy to which a mask may be accurately fitted. Although there is thus no *a priori* reason for any error from this source being confined to obese people, in a series of 28 experiments the R.Q. was determined by means of a mouthpiece and noseclip, with which any leak is practically impossible. The average value was 0·014 higher than the value obtained from the same subjects, on the same occasions, with the mask. Nearly all the patients complained of greater discomfort from the mouthpiece and noseclip, and this in itself is probably sufficient to account for the slight discrepancy between the two methods—especially as the average B.M.R. was also 2·5 % higher with the mouthpiece than with the mask. In any case, a difference of 0·014 is insufficient to bring many of the low values of the R.Q. within the normal range.

(2) *Leakage from the Douglas bags.* The Douglas bags were filled with expired air from a spirometer, itself rigorously tested for leaks, samples being removed during the filling. Ten minutes later—the maximum time which ordinarily elapsed between their being filled and measured—samples were again removed, and still further samples were obtained after 30 minutes. On each occasion three samples were taken, and analysis was done in duplicate. The results showed that in 10 minutes there was insufficient leakage to cause appreciable fall in the R.Q., though in 30 minutes enough CO_2 had been lost to lower the R.Q. by 0·01 to 0·02. Evidently our procedure of emptying the bags within 10 minutes of their being filled was safe but must be rigorously adhered to. In confirmation of this was the fact that samples of expired air collected from the side-tube of the Douglas bag during the course of actual respiration experiments gave, on analysis, results practically identical with those from the same bag after standing for 10 minutes.

(3) *The analytical technique.* The accuracy of the analytical technique was proved by satisfactorily accurate determinations of the R.Q. of absolute alcohol

combusted in glass vessels. Of course, samples of the laboratory air were analysed each day, and no experimental work with the apparatus was undertaken until such analysis gave satisfactory results.

(4) *Errors due to the subject.* Errors in the observed R.Q. may, of course, be due to the subject not being in a strictly basal condition, though any such errors would probably tend to raise rather than to lower the R.Q. In all our subjects, however, the strictest precautions were taken to ensure basal conditions, and they were all well habituated to the technique before serious observations were started. Careful observations were made of the subject's respiratory movements before and during the experiment, and we are satisfied that in all the results recorded there was no error from alteration in the type of respiration during the collection of the sample.

The R.Q. of normal individuals.

Any systematic error, leading to a general lowering of all our values of R.Q. was unlikely in view of the results obtained in routine hospital determinations. These, however, were not from healthy individuals, and we therefore thought it desirable to compare our value for the R.Q. of normal healthy subjects with that given by other workers.

Seven normal healthy women students, accustomed to breathing through a mask, were chosen for this experiment. After an ordinary mixed supper in hospital at 7 p.m., the subject remained fasting and in bed from 9 p.m. until the test was made shortly after 8 a.m. the next morning. Two determinations of the B.M.R. were made on each subject (all were within normal limits) and four samples of air were analysed from each bag. The quadruplicate analyses agreed, and an average basal R.Q. of 0·797 was obtained over the whole series, the lowest individual value being 0·760.

Du Bois [1927] states that after ordinary mixed fare the normal basal R.Q. lies between 0·80 and 0·84, and an average quotient of 0·83 was calculated by Joslin [1923] from the results obtained in 236 out of 239 normal individuals in the Harris and Benedict series [1919]. On the other hand, a study of 61 readings in Benedict's famous Squad A [Benedict *et al.*, 1919] shows an average R.Q., when the subjects were on normal diet, of 0·795—a figure which is identical with our own. We may mention that all our subjects were under the strictest possible basal conditions at the time of the test. Under these circumstances we believe that our mean value for the R.Q. in normal subjects is correct, and does not indicate any general artificial lowering of our determinations such as would invalidate the results obtained from the obese subjects.

General treatment of the subjects.

On entering the ward for the investigation of metabolism, the subject was at first given what would normally be a maintenance diet of about 2500 Cals. Most of the subjects, however, had been accustomed to eating considerably

more than this, and even so were only maintaining their weight and not increasing it. To them, therefore, the supposedly maintenance diet was in reality sub-caloric, and they almost invariably lost weight on it. This diet was continued for some days during which repeated determinations of the basal B.M.R. and R.Q. were made, and the patient was thus habituated to the technical procedures.

The patient was then given a diet of, usually, 1000 Cals. This was normally made up of 66 g. of protein, 38 g. of fat, and 100 g. of carbohydrate, but occasionally the amount of carbohydrate was reduced with compensatory addition of fat. Finally, the effect of the sub-caloric diet was reinforced by administration of thyroid extract.

Throughout the whole of this time, along with frequent determinations of the B.M.R. and R.Q., and estimations of the urinary nitrogen, the presence or absence of acetonuria was noted daily. In a number of instances, when acetone was present, the total excretion of ketones was determined quantitatively, but was never found to exceed 0·5 g. daily.

In this note on the treatment of the subjects, as in the discussion which follows, we have included only those points relevant to our present purpose. A fuller account, from the therapeutic aspect, is given by Lyon and Dunlop [1932].

Results and discussion.

Table I gives the results of the determinations of the B.M.R. and R.Q. in a number of typical cases, and the total number of determinations has been summarised in Table II.

The average of 34 estimations of the R.Q. in obese persons receiving a diet of 2000 to 2500 Cals. is 0·755, which is significantly below the normal average. As has been pointed out, however, this diet, normally sufficient for maintenance, is actually sub-caloric for the obese subject who was losing weight on it.

When the patient was receiving a more definitely sub-caloric diet of 1000 to 1200 Cals. daily, the average basal R.Q. was still lower—0·721—but still just within the range of normal metabolism. Addition of thyroid extract made little difference to the average basal R.Q., but it is evident, from the fact that in the thyroid group relatively few values below 0·70 were obtained, that there is, in individual cases, a tendency for the R.Q. to rise when thyroid is administered (*cf.* records of individual patients in Table I). Indeed, many of the unduly low values, which depress the average of the thyroid group were probably obtained before the full effect of the hormone had appeared.

It is possible that in experiments of short duration the observed R.Q. bears no true relation to the oxidative metabolism actually proceeding, even though it accurately represents the gas exchange during the experimental period. Theoretically, this must be the case in an experiment of infinitesimally short duration, though in a long one of, say, 24 hours the R.Q. undoubtedly represents the net result of metabolism during that time. Hence it is necessary to enquire

Table I.

Case	Date	Diet, Cals.	Acetonuria	R.Q.	O_2 used per hour, litres	B.M.R.
Mrs A. S.	27. xi. 31	2700	–	0·77	20·85	+18
	1. xii. 31	.	–	0·78	20·08	+14
	3. xii. 31	1000	–	0·74	20·62	+18
	7. xii. 31	.	–	0·70	19·74	+11
	10. xii. 31	.	–	0·68	19·59	+10
	14. xii. 31	.	+	0·78	18·40	+ 5
	17. xii. 31	.	–	0·70	20·19	+13
	22. xii. 31	.	+	0·69	18·55	+ 4
	24. xii. 31	.	–	0·69	18·63	+ 5
	28. xii. 31	.	–	0·77	17·80	+ 3
	31. xii. 31	.	–	0·67	18·75	+ 5
	4. i. 32	.	–	0·69	20·25	+15
	8. i. 32	.	–	0·76	19·37	+11
	11. i. 32	.	+	0·73	20·31	+ 8
	14. i. 32	.	–	0·75	18·35	+ 6
	18. i. 32	.	–	0·69	18·01	+ 4
	21. i. 32	.	–	0·70	19·47	+13
	22. i. 32	.	Trace	0·74	18·16	+ 6
	25. i. 32	.	Trace	0·70	18·55	+ 9
	28. i. 32	.	–	0·72	18·65	+ 9
	29. i. 32	.	Trace	0·75	16·81	+ 3
	5. ii. 32	.	–	0·69	16·17	– 5
	8. ii. 32	.	–	0·74	17·45	+ 4
	13. ii. 32	.	–	0·68	17·56	+ 4
Mrs M.	5. xii. 30	1800	–	0·77	14·80	– 7
	8. xii. 30	2000	–	0·78	14·63	– 4
	14. i. 31	1000	–	0·74	14·47	–11
	21. i. 31	.	Trace	0·74	13·03	–11
	28. i. 31	1000 + thyroid	–	0·76	16·75	+15
	3. ii. 31	.	–	0·76	17·92	+18
	10. ii. 31	.	–	0·76	.	+15
	12. ii. 31	1000	–	0·68	18·32	+19
	13. ii. 31	.	–	0·66	17·83	+17
	14. ii. 31	.	–	0·64	16·10	+ 9
Mrs O.	27. ix. 28	1000	–	0·75	15·84	+14
	3. x. 28	2000	–	0·76	.	– 8
	10. x. 28	.	–	0·81	10·76	–19
	18. x. 28	800	–	0·74	13·30	– 1
	25. x. 28	1000	–	0·76	12·90	– 3
	31. x. 28	.	–	0·77	13·44	+ 1
Miss M.	12. viii. 29	2000	–	0·77	15·76	– 9
	30. viii. 29	1000	–	0·76	.	+ 4
	31. viii. 29	.	–	0·69	17·93	+ 4
	22. x. 29	.	+	0·68	18·65	+13
	10. ii. 30	.	–	0·67	13·98	– 4
	11. ii. 30	.	–	0·67	12·76	– 3
	18. ii. 30	1000 + thyroid	–	0·75	15·38	+13
	10. iv. 30	1000	–	0·67	18·03	– 4
	11. iv. 30	.	–	0·67	15·42	– 6
	18. v. 30	1000 + thyroid	–	0·75	.	+14

Table II.

Calorific value of diet	Number of determinations	Average R.Q..	Range	Number below 0·70	
					%
2000 and above*	34	0·755	0·67–0·86	3	9
1500–2000	30	0·722	0·65–0·86	11	36
1000–1200 standard	117	0·721	0·63–0·87	51	44
1000 low carbohydrate	15	0·678	0·63–0·76	12	80
1000–1200 + thyroid	70	0·727	0·66–0·84	14	20

* These diets were sub-caloric for the patients receiving them, and the patients were losing weight.

whether, in our experiments of 6 minutes, the observed data (granting their accuracy) can be accepted as expressing the oxidative metabolism during that period. Our experimental evidence bearing on this point falls into three categories. Firstly, as a routine measure, two successive independent determinations of the R.Q. were made at an interval of about 10 minutes, but without any attempt at regulating the interval exactly. Secondly, in a number of cases, the interval between the two determinations was exactly half an hour. Lastly, in a few cases, four independent determinations were made, one after the other, the patient wearing the mask throughout. These three procedures would expose any haphazard variations, or any accidental tendency in the routine treatment to hit off a possible periodicity in the R.Q. In the vast majority of the results the successive estimations of the R.Q. and of the oxygen consumption agreed very closely, and in only a very few cases was there any marked and unaccountable discrepancy. Since the agreement of duplicate or quadruplicate determinations was the rule, it could not be ascribed to coincidence, and, consequently, the 6-minute determinations may justifiably be regarded as giving a true expression of the metabolism at that time. Nevertheless, it must always be realised that reliance on the technique of indirect calorimetry is dangerous, and that our results, together with the interpretation to be placed on them, must fall if any technical error (which, however, we have failed to detect) can be shown to account for them.

Our discussion and tentative interpretation of our results is based on the supposition that individual determinations of the R.Q. are reliable, and that therefore the subjects were at times exhibiting an abnormal type of metabolism. (It is to be noted that patients varied in the results they yielded; some gave a low R.Q. more frequently than others, though none invariably gave results below 0·70.) The fact that the averages in Table II are so near the normal minimum interests us only as showing that, with a distribution similar to that of healthy individuals, many values must have been below 0·70—if the average is correct. Of that there is no reason to doubt in view of the value obtained for the average R.Q. in normal healthy subjects.

An R.Q. below 0·70 can only mean that oxygen is being used without production of a corresponding amount of carbon dioxide—*i.e.* for some incomplete oxidation. It has already been pointed out that the ketonuria occasionally observed was inadequate to account for the low values of the R.Q. even in the patients who showed it. It might be objected, however, that in the special case of the obese subject, ketosis might occur without corresponding ketonuria, the excess keto-acid being stored in the fatty tissues by virtue of preferential solubility in fat. According to this hypothesis, ketosis would occur during starvation without ketonuria but with a low R.Q., and after a carbohydrate-containing meal the stored ketones would be oxidised with production of an abnormally high R.Q. We have measured the R.Q. in our obese subjects at regular intervals for 4 hours after a meal, but have never found evidence of this occurring.

We have, moreover, shown by direct *in vitro* experiments that there is no preferential solubility of ketone substances in fat as compared with Ringer's solution or serum. In a typical experiment, Ringer-Locke solution containing 60 mg. of acetoacetic acid per 100 cc. (p_H 7·40) was shaken with an equal volume of olive oil for 90 minutes. The two layers were then separated by centrifuging, and acetoacetic acid was determined by Van Slyke's method in the aqueous layer and in the original solution. The results were: before shaking, 59·75 mg. acetoacetic acid per 100 cc.; after shaking, 60·50 mg. per 100 cc. A similar experiment with acetone (*a priori* more likely to show solubility in fat than sodium acetoacetate) gave a similar result: before shaking, 244 mg. acetone per 100 cc.; after shaking, 216 mg. per 100 cc.

The only other incomplete oxidation of which one can conceive as taking place in significant amount is the conversion of protein or fat into carbohydrate. Evidence from the urinary D/N ratio of 3·65 in severe diabetes and in depancreatised or phlorhidzinised dogs indicates that about 58 g. of carbohydrate may be formed from 100 g. of protein. This figure is derived from study of the fate of ingested protein, of which a small part of the nitrogen is lost in the faeces. When the body is metabolising its own protein exclusively, so that the whole of the nitrogen appears in the urine, this figure may be slightly altered. However, any error introduced by its use is a very small one. Even if, on this basis, it be supposed that all the protein metabolised is converted to carbohydrate which is stored, it appears that the R.Q. for the remainder is frequently below 0·70. This, in the absence of incomplete oxidation of fatty acid to keto-acids, and after allowance for the possible conversion of glycerol to glucose, strongly suggests the formation of carbohydrate from fatty acid.

The calculation to which the experimental data have been submitted is shown below, and in Table III are given the results for a number of abnormally low values of R.Q., all obtained in absence of ketonuria. The formulae apply to the usage of oxygen and production of CO_2 on the assumption that maximal production of carbohydrate from protein is taking place and that at least as much carbohydrate as this is being stored. Hence if, on solution of the equations, y is zero, the gain of carbohydrate to the body is equal to the production from protein, if y is negative, less than this amount is being stored, if y is positive, additional sugar is being produced and stored from fat. There is no assumption that carbohydrate is not being oxidised—merely that with abnormally low R.Q.'s it is being produced to a greater extent than it is being burnt. Since it is highly probable that some carbohydrate is always being oxidised, a zero value of y still indicates the probability of formation of sugar from fat, and even with positive values of y only the net gain, and not the total production of carbohydrate is calculable.

The values for the non-protein R.Q. in Table III are evidently well below 0·707, even when full allowance is made for maximal carbohydrate storage from protein. If further allowance is made for conversion of glycerol to

Table III.

Case	Date	Total R.Q.	For 1 hour, fasting: Litres O_2	Litres CO_2	g. urinary N_2	Non-protein R.Q. with normal protein oxidation	Assuming maximal carbohydrate from protein: Non-protein R.Q.	g. fat. completely oxidised	g. fat. converted to carbohydrate
J. McW.	14. v. 31	0·670	14·76	9·89	0·470	0·634	0·673	6·21	1·13
	22. v. 31	0·675	12·31	8·32	0·422	0·643	0·680	5·19	0·77
	8. vi. 31	0·650	13·56	8·82	0·247	0·631	0·651	5·80	1·77
V.	17. vii. 29	0·666	14·27	9·51	0·410	0·638	0·668	6·04	0·89
	19. vii. 29	0·656	14·31	9·39	0·415	0·624	0·652	5·95	1·58
	22. vii. 29	0·678	13·37	9·07	0·380	0·652	0·683	5·78	0·77
A. C.	29. vii. 29	0·668	19·48	13·00	0·450	0·645	0·670	8·42	1·68
	1. viii. 29	0·650	20·28	13·20	0·465	0·626	0·652	8·55	2·56
	20. viii. 29	0·679	19·79	13·42	0·445	0·653	0·683	8·72	1·23
M.	10. ii. 30	0·670	13·98	9·37	0·260	0·653	0·672	6·17	1·14
	11. ii. 30	0·670	12·76	8·55	0·245	0·652	0·672	5·62	1·04
J. H.	12. iii. 30	0·677	17·24	11·67	0·510	0·649	0·683	7·41	1·04
Mn.	12. ii. 31	0·680	20·13	13·67	0·448	0·659	0·682	8·90	1·19
	13. ii. 31	0·665	19·49	12·95	0·482	0·640	0·667	8·35	1·80
	14. ii. 31	0·641	17·22	11·04	0·408	0·614	0·641	7·12	2·54
H.	12. ii. 31	0·683	18·32	12·51	0·448	0·662	0·687	8·09	0·88
	13. ii. 31	0·664	17·83	11·84	0·430	0·640	0·666	7·64	1·67
H. K.	17. ii. 31	0·696	15·81	11·00	0·650	0 661	0·704	6·73	0·07
	25. ii. 31	0·664	12·14	8·06	0·479	0·620	0·632	4·67	1·06
	5. iii. 31	0·700	12·47	8·73	0·576	0·660	0·711	5·25	0·00
E. S.	9. iii. 31	0·682	17·51	11·94	0·684	0·644	0·688	7·34	0·79
	10 iii. 31	0·680	16·22	11·02	0·690	0·636	0·685	6·68	0·79
	19. iii. 31	0·627	15·52	9·74	0·450	0·590	0·625	6·14	2·76
	27. iii. 31	0·630	16·22	10·21	0·390	0·601	0·628	6·56	2·71
A. S.	31. xii. 31	0·670	18·75	12·56	0·304	0·655	0·671	8·33	1·58
	18. i. 32	0·690	18·01	12·42	0·299	0·677	0·693	8·25	0·67
	13. ii. 32	0·680	17·56	11·94	0·320	0·665	0·683	7·87	1·04

Example of calculation.

I. *Data.*

(*a*) The figures given by Lusk [1928, p. 69] have been used for the consumption of oxygen and production of carbon dioxide during the normal oxidation of fat and carbohydrate:

1 g. fat requires 2·019 litres O_2 and gives 1·427 litres CO_2.
0·9 g. glucose, or 1 g. glycogen, requires 0·828 litres O_2 and gives 0·828 litres CO_2.

(*b*) In dealing with protein, Lusk's figures for the elementary composition of meat protein have been used, but it has been assumed that as body protein is being metabolised there is no loss in the faeces. This assumption may not be correct, but if anything it weighs the balance against the conclusion of fat conversion to carbohydrate, since it increases slightly the amount of protein carbon available for similar conversion.

	C g.	H g.	O g.	N g.
100 g. meat protein contain	52·88	7·27	22·68	16·65
Of this there appear in the urine	9·41	2·66	14·10	16·65
For respiration	43·17	4·61	8·58	—
Deduct water equivalent	—	1·07	8·58	—
	43·17	3·54	—	—

Hence 100 g. protein require 100·4 litres O_2 and give 80·6 litres CO_2.
Thus with normal metabolism,

1 g. urinary N ≡ 6·005 g. protein ≡ 6·025 litres O_2 used ≡ 4·84 litres CO_2 produced.

With maximal conversion of protein to carbohydrate,

1 g. urinary N = 3·65 g. glucose, which, on oxidation, would use 2·72 litres O_2 and give 2·72 litres CO_2.

Hence, with this glucose stored,

1 g. urinary N = 3·305 litres O_2 used, and 2·12 litres CO_2 produced.

(R.Q. = 0·641.)

(*c*) For the hypothetical conversion of fat into carbohydrate, the fat is taken as having the composition of glyceryl dioleate monostearate, $C_{57}H_{106}O_6$, which represents fairly closely the average composition of body fat. Small differences in composition have, however, no appreciable effect on the result. It is further assumed that all the carbon of the fat molecule is retained in the carbohydrate molecule, the change being represented as:

$$2C_{57}H_{106}O_6 + 47O_2 = 19C_6H_{10}O_5 + 11H_2O.$$

According to this equation:

1 g. fat requires 0·594 litres O_2, produces no CO_2, and yields 1·736 g. of glycogen.

II. *Formulae.*

From these data, formulae are derived as follows:

Let observed O_2 usage over a given period $= a$ litres
" CO_2 production over a given period $= b$ litres
" urinary nitrogen over the same period $= c$ g.

With maximal carbohydrate production from protein,

O_2 for non-protein metabolism $= a - 3{\cdot}305\,c$.
CO_2 from non-protein metabolism $= b - 2{\cdot}12\,c$.

Let x g. fat be oxidised completely,
y g. fat be converted to carbohydrate.

Then O_2 used $= 2{\cdot}019x + 0{\cdot}594y = a - 3{\cdot}305\,c$.
CO_2 produced $= 1{\cdot}43x = b - 2{\cdot}12\,c$.

Whence
$$x = \frac{b - 2{\cdot}12\,c}{1{\cdot}43}.$$
$$y = \frac{a - 1{\cdot}41\,b - 0{\cdot}31\,c}{0{\cdot}594}.$$

carbohydrate, the non-protein R.Q. may be as low as 0·695, the fatty acid being completely oxidised. Values below this figure must then indicate incomplete combustion of fatty acid. The figures given in the last two columns of Table III represent the amounts of fat oxidised and stored as carbohydrate, on the assumption that such a conversion takes place, and that its equation is that postulated by Zuntz. Other calculations, based on other hypothetical equations, such as that of Chauveau, give higher figures for the amount of fat converted to carbohydrate, since they involve the simultaneous production of a little CO_2, and, in the case of Chauveau's equation, an R.Q. of 0·29 for the conversion. No stress is laid on the figures given in these two columns; they are merely intended to show that if conversion of fat into carbohydrate is taking place, it is proceeding to an appreciable extent, and to emphasise the difference between the observed R.Q. and the minimal value possible on the assumption of normal and complete oxidation of fatty acid.

On the assumption of carbohydrate formation from fat with conservation of all the carbon, Table III shows that the glucose production accounts for about 1/10 to 1/5 of the fat metabolised. These amounts are appreciable, but even if they be added to the amounts supposed to be formed from protein, they would not cause a very rapid increase in the glycogen reserves, and could therefore be conceived of as continuing for some hours.

It is obvious that such a storage of carbohydrate cannot continue indefinitely. Accordingly, it is found that after a meal, with the subject still resting, the R.Q. rises above 0·70, sufficiently to indicate usage of carbohydrate during this period. This indicates that the carbohydrate storage mechanism, if it exists at all, is essentially one called into action only during a fast. It is also noteworthy, that even with the patient continuously on the same sub-

caloric diet, the fasting R.Q. is not invariably below 0·70. There is a tendency for the R.Q. to rise and fall in a periodic way, as if the need for storage appeared when the carbohydrate reserve had fallen below a certain level, and disappeared when, by storage from food or synthesis, the reserve had been built up to some higher level.

If, however, it be objected that the actual storage of carbohydrate is so improbable that the assumption of its occurrence must be rejected, we are faced with still greater difficulties, for then we must assume that protein is being oxidised with its normal R.Q. of 0·806. This, of course, leads to still lower values for the non-protein R.Q.—values sometimes below 0·60—which cannot possibly be explained on any known hypothesis.

Without going into the whole of the vexed and still controversial question of the possibility of carbohydrate formation from fat—a question well reviewed by Rapport [1930]—we may mention a few points bearing directly on our interpretation of our experimental data. The obese person whose reserves consist predominantly of fat is evidently more likely than the normal individual to exhibit the phenomenon of fat conversion into carbohydrate under sub-caloric dietary conditions. Moreover, if we reject arguments in favour of such a conversion in diabetes based on observations of abnormally high D/N ratios, there is evidence that exogenous fat can be turned into carbohydrate in animals whose carbohydrate stores have been depleted by a period of starvation, and in which, therefore, the conditions are analogous to those obtaining in our patients. Similarly, Jost [1931] has recently observed increased sugar formation on perfusion of glycogen-poor livers with fatty acids and phosphate. It is significant, too, that in our experiments there was a tendency for a low R.Q. to appear more readily the smaller the amount of carbohydrate in the diet. The absence of ketonuria in our experiments may be reasonably explained by the assumption that part of the fat was oxidised *via* carbohydrate, thus affording a sufficiency of ketolytic material. Such a mechanism may also be the explanation of the fact, known from clinical observation, that fat diabetic subjects show less tendency to ketosis than thin ones.

In conclusion, therefore, we believe that our experimental results afford evidence that in the undernourished obese subject, fat is converted into carbohydrate. This conclusion is, however, tentative, inasmuch as it depends on a technique open to criticism, and becomes liable to revision if and when any more probable mechanism for the storage of oxygen is suggested. Valuable confirmatory evidence of the correctness of our hypothesis might be obtained from measurement of the actual heat production simultaneously with the respiratory exchange. Such a measurement would enable the calculated heat production to be compared with the actual. This technique was, however, beyond our means. Our interpretation does not of course imply that a conversion of fat into carbohydrate takes place ordinarily as an obligate step in the metabolism of fat; but simply that in certain abnormal circumstances the animal body is capable of it. Indeed, experiments on muscular exercise

[*cf.* Stewart, Gaddie, and Dunlop, 1931] indicate that when fat is used as a source of energy for external work, it is directly oxidised and is not first converted into sugar. In other words, the auxiliary verb for the conversion of fat to carbohydrate is, at most, "may" and not "must."

Summary.

1. In a series of 34 observations on the R.Q. of obese subjects on a sub-caloric diet of 2000 or more Calories loss of weight occurred and the average R.Q. was 0·755. Of these, 3 were below 0·70.

2. When the diet was reduced to 1000 to 1200 Calories the average R.Q. in 117 observations was 0·721 with 51 below 0·70.

3. When the carbohydrate content of these latter diets was reduced, but the total calorific value maintained, the average R.Q. fell to 0·678, 12 out of 15 being below 0·70.

4. Administration of thyroid to the subjects on 1000 to 1200 Calories diet produced a slight increase in the R.Q., the average of 70 determinations being 0·727, with only 14 below 0·70.

5. Ketonuria was usually absent when these observations were made and when present was only slight.

6. These results are discussed with respect to the conversion of fat to carbohydrate.

The thanks of two of us (D. M. D. and C. P. S.) are due to the Medical Research Council for part-time grants.

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CXXXI. THE REACTION OF FISH-LIVER OILS WITH ANTIMONY TRICHLORIDE.

(PRELIMINARY COMMUNICATION.)

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(*Received June 7th, 1932.*)

It is a well-known fact that fish-liver oils and other products containing vitamin A give a blue colour reaction with antimony trichloride, the intensity of which, within certain limits and under definite conditions regarding the concentration of the solutions, runs parallel with the biological activity of the oil. As yet the question whether the substance reacting with $SbCl_3$ is the vitamin itself or an accompanying compound has not been definitely settled.

The purpose of the present communication is to report some experiments bearing on this problem.

It is difficult to obtain the blue product resulting from the interaction of antimony trichloride and liver oil in quantity and condition suitable for examination. As is well known, the blue colour is exceedingly unstable. It reaches its maximum intensity some few seconds after the mixing of the solution of oil in dry chloroform with the reagent (a saturated solution of antimony trichloride in chloroform). After about 60 seconds the colour has already begun to fade, and it disappears completely in the course of the next 15 minutes.

A number of other dry, inorganic salts[1] were tried with no evident advantage. With concentrates of vitamin A, a blue or bluish colour was always obtained, but the colour was no more stable and often fainter than that produced by the antimony trichloride. This happened especially with salts only slightly soluble in chloroform. As is well known, the addition of water makes the colour disappear almost instantaneously; the formation of the blue colour is also prevented by acetone, ether, acetaldehyde and various alcohols. The stability of the blue colour is largely influenced by even small temperature changes, an increase of 10° above the ordinary room temperature makes the colour fade more quickly, while a corresponding fall in temperature has the opposite effect. An intense cooling increased the stability of the blue colour for a considerable time. At − 35° it persisted for at least some hours, and after subsequent cooling to − 50° a heavy blue oil was precipitated on the bottom of the vessel.

[1] $AlCl_3$, $AlBr_3$, $AsCl_3$, $BiCl_3$, $FeCl_3$, $SbBr_3$, $SbCl_5$, $SnCl_4$, $SnBr_4$, $TeCl_4$, $TiCl_4$, $ZnCl_2$, $ZnBr_2$

It was therefore decided to work at a low temperature, and then to decompose the blue oil produced according to the following hypothetical equation.

1. Chromogenic substance (Vitamin A?) + $SbCl_3$ = Blue product.
2. Blue product + H_2O = Chromogenic fraction + SbOCl + 2HCl.

Liver oil of halibut was chosen as a convenient raw material. It contains 50–100 times more vitamin A than cod-liver oil, and at the same time it is relatively poor in vitamin D. The saponification was carried out in the usual way, the oil being boiled with an excess of 5 % alcoholic KOH. The saponification was repeated until a sample of the product was practically free from glycerides. The product was next dissolved in methyl alcohol, cooled down to about − 5° and the crystalline cholesterol filtered off. After evaporation of the methyl alcohol the residue consisted of a reddish-yellow oil, which showed an antimony trichloride value of 13,500 blue units (Lovibond)[1], and when tested on rats proved active in daily doses of 0·002–0·003 mg. All experiments were carried out in an atmosphere of nitrogen or carbon dioxide. The distillations were likewise performed in indifferent gases or *in vacuo* and at a low temperature.

Preparation of the blue product.

15 g. of the unsaponifiable fraction were dissolved in 600 cc. of dry chloroform, and the solution was at once cooled down to − 35° in a cooling mixture of carbon dioxide, alcohol and glycol. About 1300 cc. of a saturated solution of $SbCl_3$ in dry chloroform were added under constant stirring, care being taken that the temperature did not exceed − 30°. On subsequent cooling to − 55° a heavy blue oil was precipitated, from which the faintly coloured chloroform solution could be decanted. To this oil pieces of ice or an ice-cold mixture of water and 10 % KOH (for the neutralisation of the possibly harmful hydrochloric acid) were added under constant stirring until the blue colour disappeared. Then the mixture was extracted with benzene. The benzene solution was filtered, washed with water and subsequently dried with sodium sulphate and the benzene distilled off *in vacuo*. The residue weighed 11·25 g. It was a highly unsaturated reddish-yellow oil, which we may call "the chromogenic fraction," as it still reacts with antimony trichloride. The antimony trichloride value is however only 4000–5000 blue units, *i.e.* about 1/3 of the colour value given by the original concentrate. In doses of 0·01–1 mg. per day it showed no biological effect (23 rats). On the addition of ethyl alcohol to the oil an amorphous yellow powder was precipitated, the blue value ($SbCl_3$) of which was variable, *viz.* 1700–8700 Lovibond units. The reason apparently was that the powder, when dried and exposed to the air, underwent a rapid transformation and became almost colourless. The freshly prepared product had an unsharp melting-point between 160°–170°. It was easily soluble in benzene,

[1] Measured with the Rosenheim-Schuster colorimeter according to the method described by Rosenheim [1927], the highly active preparations being taken into consideration and the readings being made half a minute after the addition of the $SbCl_3$ solution.

chloroform and ether with an intense yellow colour. In daily doses of 0·002–0·02 mg. it was biologically inactive (24 rats). When the chromogenic fraction was treated with $SbCl_3$ at low temperature and the reaction product decomposed with water a chromogenic substance was again formed.

The non-chromogenic fraction.

On addition of cold light petroleum (− 45°) to the faintly coloured chloroform solution mentioned above, the last traces of the blue product were precipitated. To the separated solution which was almost colourless, ice or cold aqueous KOH was added under constant stirring. The aqueous layer was removed in a separating funnel, the chloroform-light petroleum solution was dried with sodium sulphate and evaporated *in vacuo* on a water-bath. The residue, a faintly yellow oil, only gave a slight coloration with antimony trichloride and in daily doses of 0·05 and 0·4 mg. the biological effect was nil (6 rats).

The results of the biological experiments seem to justify the conclusion that the antimony trichloride reaction is not specific for vitamin A. This is in agreement with Euler and Euler [1930]. It was shown by these authors that a series of carotenoids devoid of any biological effect (vitamin A), gave a blue colour with antimony trichloride. In addition to its biological action, vitamin A, according to several investigators, is characterised by a broad, selective absorption in the ultra-violet region of the spectrum, with a definite maximum at 328 $m\mu$. It was accordingly desirable to make a spectrographic comparison of the chromogenic fraction and the liver oil concentrates of *Hippoglossus hippoglossus*. It was found that the exceedingly active preparations of the halibut-liver oil showed a very distinct absorption band in the ultra-violet range at 326–327 $m\mu$. This band was not observed in the chromogenic fraction. The characteristics of the absorption spectrum of this fraction seem to be the following.

A strong absorption in the ultra-violet from 390 $m\mu$ towards shorter wavelengths, with bands at 370, 351 and 300–290 $m\mu$. The absorption curves obtained with some of the preparations are presented in Fig. 1 and Fig. 2.

The absorption spectra given by the two blue products ($SbCl_3$ + concentrate, or chromogenic fraction) were compared both spectrographically and spectrophotometrically. As the spectrophotometric measurements in the Albrecht apparatus gave only approximate values, the more exact spectrographic data are alone reported. The spectrogram of the blue product, obtained from the concentrates, showed two absorption bands, at 622·5 (621·1) and 590 $m\mu$ respectively. The first was very sharp and its position easy to place, the other was less distinct and the wave-length in this case could hardly be determined with any degree of accuracy. In some preparations of the blue product the second band was localised at 575 $m\mu$. In contrast with the concentrate the chromogenic fraction is partially insoluble in alcohol. The blue product obtained from the chromogenic fraction exhibits two absorption bands, differing from those

seen in the blue product from the concentrate. In the case of the chromogenic fraction the maximum absorption is at 590 (586·8) $m\mu$ and 550–522 $m\mu$. Concerning these bands it is likewise to be noted that the first is the most

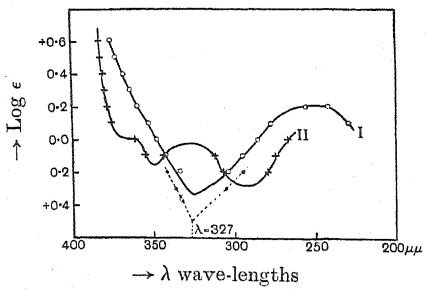


Fig. 1.

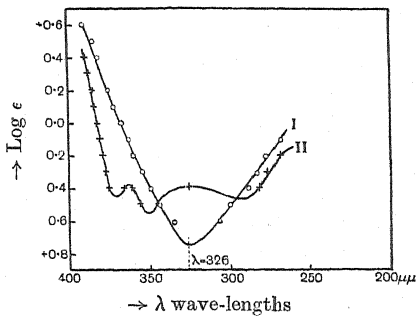


Fig. 2.

Absorption curves for the concentrate (I) and the "chromogenic fraction" (II).
The positions of the points marked • were determined photometrically, the others visually.
$\epsilon = 1/d \log I_0/I$.
I_0 = intensity of the incident light.
I = intensity of the transmitted light.
d = thickness of the cell in cm.

distinct. The positions of some of the absorption bands in the spectrograms mentioned above were determined both visually and photometrically. The values obtained with the photometer are given in parentheses.

The maxima of the most distinct bands can be localised with an accuracy of about ± 1–2 $m\mu$; as to the weaker bands the position cannot be determined with any great degree of accuracy owing to their lack of sharpness.

Discussion.

The spectrographic results obtained with the vitamin A concentrates (of halibut-liver oil) are in close agreement with the findings of Morton, Heilbron and Thompson [1931], Heilbron, Gillam and Morton [1931], and Coward, Dyer, Morton and Gaddum [1931]. According to several investigators, the ultra-violet absorption with its maximum near 328 $m\mu$ has been observed in all the biologically active preparations examined, and seems to be characteristic for vitamin A. By treating the vitamin concentrates with $SbCl_3$ and adding water to the blue compound thus formed, the vitamin is rendered inactive.

The addition of water results in the formation of a yellow oil, which I have called the "chromogenic fraction." With antimony trichloride this fraction gives a strong blue colour, which cannot be attributed to vitamin A itself, but must be due to yet another chromogen. As mentioned by Heilbron, Gillam and Morton [1931] the concentrate of halibut-liver oil probably contains two chromogens.

One of these chromogens is supposed to cause the absorption at 328 $m\mu$, and that at 583 $m\mu$ of the blue product, while the other chromogen in the colour reaction is responsible for the absorption band at 620 $m\mu$. The physico-chemical characteristics of vitamin A, according to these authors, are as follows: "a colourless or pale yellow substance exhibiting selective ultra-violet absorption free from fine structure and absorbing maximally at or near 328 $m\mu$ and giving with antimony trichloride a coloured substance, characterised by a maximum in the yellow between 580 and 590 $m\mu$, whilst the molecular extinction coefficients at 328 and 580–590 $m\mu$ expressed in terms of vitamin concentration should be approximately equal."

Comparing my own results with the findings of Heilbron, Gillam and Morton [1931], it is striking that my highly active concentrates exhibit with antimony trichloride a decided absorption maximum at 622 $m\mu$, while, on the other hand, the inactive preparation ("chromogenic fraction") in the colour test does not show this band, but exhibits a strong absorption in the neighbourhood of 590 $m\mu$ and a fainter one at 551 in the green part of the spectrum.

While Heilbron, Gillam and Morton state that oxidation of certain liver oils, containing vitamin A, leads to an increase in the intensity of the absorption at 606, or 620 for concentrates, I have observed that a destruction of vitamin A subsequent to treatment with antimony trichloride and water abolishes this band (622). On the other hand, the absorption at 590 $m\mu$ is exceedingly pronounced. I must conclude therefore that an absorption band at 590 $m\mu$ produced by the interaction of antimony trichloride and the "chromogenic fraction" cannot be attributed to vitamin A. It must however be taken into account that so powerful an influence on the inner structure of the substance, as a treatment with $SbCl_3$ and water must probably exert, may also show itself in a displacement of the absorption bands. From that point of view it is possible that the chromogenic nature of the concentrates after the treatment with $SbCl_3$ and water may be due to the decomposed vitamin.

Summary.

A certain parallelism exists between the biological activity and the intensity of the blue colour given by liver oil concentrates of *Hippoglossus hippoglossus* with antimony trichloride.

At room temperature the blue colour thus formed is exceedingly evanescent, but at − 40° the reaction-product is stable for hours.

Concentrates of halibut-liver oil probably contain two chromogens, of which one is possibly identical with vitamin A, and is destroyed by treatment with antimony trichloride and water, while the other, which presumably has no connection with growth in animals, still reacts with antimony trichloride forming a blue product from which the chromogen may be regenerated by the addition of water. The absorption bands of the liver oil concentrate and the "chromogenic fraction," together with the spectra of their reaction products with antimony trichloride, are described.

I am indebted to Professor E. Poulsson for his generous help and advice during the work and I also wish to thank Professor L. Vegard and Professor E. Langfeldt of Oslo University who kindly placed at my disposal the necessary instruments for measuring the absorption spectra.

Miss Qviller and Mr Stensholt gave me their valuable assistance during some of the spectrographic measurements.

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CXXXII. AN IMPROVED AND SIMPLIFIED BENZIDINE TEST FOR BLOOD IN URINE AND OTHER CLINICAL MATERIAL.

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THE principle of the benzidine test for blood, as is well known, depends upon the peroxidase action of haematin, the benzidine being oxidised to a blue compound. The great drawbacks to the test are the instability of the hydrogen peroxide solution and its impurities. Ozonic ether is often substituted for hydrogen peroxide on account of its greater stability but of course it is much more expensive. Theoretically hydrogen peroxide is the perfect oxidising agent, but the instability of aqueous solutions, together with the impurities—acids, sulphates, phosphates, and alkali metals—which are generally present, detract considerably from its usefulness in practical laboratory work, and blank tests are always necessary to control results.

About 24 years ago Tanatar [1908] produced a solid compound of hydrogen peroxide and urea which was stable, neutral and ashless, and free from inorganic impurities, and under the registered name of "Hyperol" it is available on the market at a low price. It contains 35 % of hydrogen peroxide, remarkably pure—the only impurity being the merest trace of citric acid which, however, is insufficient to affect titrations with methyl orange—and perfectly stable. It only begins to decompose when heated to 60°, and the decomposition results in the formation of oxygen, water and urea.

The stability, purity and strength of hyperol render it a most useful, reliable and powerful oxidising agent. As a powder it is easy and convenient to handle and will keep indefinitely provided it is not brought into contact with moisture. Containing 35 % of hydrogen peroxide, it is much more powerful than the 10 volume (3 %) aqueous solution which is generally used in laboratories.

In the benzidine test for blood hyperol can be used, instead of the hydrogen peroxide solution or ozonic ether, with considerable saving of expense, rendering the test much more delicate and reliable, and so simplifying the technique that in most cases it can be done as a "spot" test.

Method as applied to urine.

1. Place a small amount of benzidine (enough to cover the tip of a pocket-knife blade) on a clean white plate.
2. Add two drops of glacial acetic acid.
3. Add one drop of urine.
4. Add a small amount of hyperol (enough to cover the tip of a pocket-knife blade).

A bright deep blue colour appears immediately if blood be present, even in a very minute trace.

Sources of error. Urine containing potassium iodide, pus or enzymes will react to this test in the same way as with the ordinary guaiacum or benzidine tests. If pus or enzymes be suspected the urine should first be heated to over 60° (boiling is not necessary) and cooled before the test is applied. No reaction with pus or enzymes will be obtained under these circumstances.

If potassium iodide be suspected add a drop of dilute sulphuric acid (10–25 %) to the blue mixture when the blue colour will disappear. Next add a drop of fresh 1 % starch solution and the blue colour will reappear if due to iodides but will fail to reappear if due to blood.

Another and simpler method of detecting iodides is to place a drop of the urine on the white plate, without any benzidine, and add a small quantity of hyperol and then a drop of 1 % starch solution. A blue colour will result.

Marked alkalinity or acidity has practically no effect on the hyperol test whereas marked alkalinity prevents the development of the reaction for blood in the guaiacum test.

Relative sensitivity of guaiacum, ordinary benzidine and hyperol-benzidine tests for blood in urine.

From a large series of tests the following results were obtained:

	Guaiacum	Ordinary benzidine	Hyperol-benzidine
Smallest quantity of blood detectable	From 1 part in 1000 to 1 in 10,000	From 1 part in 25,000 to 1 in 60,000	From 1 part in 200,000 to 1 in 260,000

Thus with the hyperol-benzidine test described one can be sure of detecting 1 part of blood in 200,000 parts of urine as compared with the 1 part in 1000 revealed with the guaiacum test. It is of course much more sensitive than the spectroscope.

The test may be applied to stomach contents, cerebro-spinal and other body fluids under the same conditions as with urine.

Blood in faeces. Rub up a small quantity of the faeces—about the size of a pea—with 2–3 cc. of water in a test-tube, and heat to over 60°, then add 2–4 drops of glacial acetic acid, shake and cool. Take one drop of the watery extract and test as with urine.

In testing faeces for blood it is much better, however, to make and test an ethereal extract as follows.

Rub up a small quantity of faeces with 2–3 cc. of water in a test-tube and heat on a water-bath to over 60° for about 5 minutes, then cool and add 2–4 drops of glacial acetic acid. Shake the mixture and then add about 3 cc. of ether and shake again. Test the separated ethereal extract in the same way as urine.

Much greater sensitivity can be obtained by decanting the ethereal extract and evaporating it on a water-bath at 60°, then treating the residue with one or two drops of distilled water and testing this moistened residue. This latter method can of course be applied to body fluids. In the case of urine it has been found that, by centrifuging the specimen and examining the deposit in this fashion, 1 part of blood can be detected in 3,000,000 parts of urine. Iodides, pus or enzymes do not interfere with the test when performed in this way.

Summary.

1. "Hyperol," a dry stable powder, containing 35 % of hydrogen peroxide, can be used as the oxidising agent in the benzidine test for blood in clinical laboratory practice.

2. By its use the performance of the test is simplicity itself and the cost is almost negligible.

3. The test so performed is thoroughly reliable and extremely sensitive.

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CXXXIII. THE ABSORPTION OF MILK PRECURSORS BY THE MAMMARY GLAND.

V. PHYSICO-CHEMICAL ASPECTS OF MILK SECRETION.

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(*Received June 9th, 1932.*)

The tendency to regard secretion as an osmotic phenomenon has, as yet, found very little expression in the particular case of the secretion of milk. Beyond the fact, reported by Van der Laan [1915] that milk is isotonic with blood, no data on the osmotic relationships of the mammary gland appear to have been established. Taylor and Husband [1922], it is true, have suggested that the flow of milk through the mammary gland is the result of osmosis induced by the elaboration of lactose. Osmosis could, however, only be induced by this means if the lactose were derived from a larger molecule such as the glycoprotein suggested by Hammond [1913]. At the present time there is no evidence for the existence of such a substance in the mammary gland, and it seems more reasonable to assume that a large part of the blood-glucose, shown by us to be absorbed by the gland [Blackwood and Stirling, 1932, I], is transformed directly into lactose. The synthesis of lactose would therefore involve a decrease and not an increase in osmotic pressure as suggested by Taylor and Husband. This fact demonstrates that definite knowledge of the nature of milk precursors is a pre-requisite for the study of the physico-chemical basis of milk secretion.

In previous papers of this series we have discussed the nature of milk precursors. In the present communication an attempt will be made to consider their absorption and subsequent fate from a physico-chemical standpoint. In such a treatment it is logical to consider first the transference of material from the blood-stream to the gland cells.

The nature of the transudate from the blood-stream to the gland cells.

The first stage in milk secretion must be the passage of a solution of milk precursors from the blood-stream to the secreting cells of the mammary gland. This might be brought about in one of two ways. (1) The intervening cell membrane might be freely permeable to all blood-plasma constituents which

[1] Carnegie Research Scholar.

are in true solution, in which case the transudate would consist of blood-plasma *minus* its colloids. (2) The cell membrane might be endowed with the property of selective absorption and thus be capable of adjusting the composition of the transudate to the requirements of the secreting cells.

The first hypothesis would imply a filtration through the cell membrane analogous to that which has been demonstrated for the glomerulus of the kidney, since the glomerular filtrate has been shown [Richards and Plant, 1917, 1921] to be identical with blood-plasma *minus* its colloids. There are, however, strong reasons for rejecting the hypothesis that a filtration of this type takes place between the blood-plasma and the cells of the mammary gland. This may be concluded from a consideration of the following experimental data.

In Table I A are given the results of analysis of blood from six lactating cows and in Table I B the analysis of milk from six cows (not the same animals).

Table I.

A. *Blood.*

Amounts mg. per 100 cc. blood			Ratios		
Glucose	NH_2-N	NaCl	Glucose	NH_2-N	NaCl
44	6·92	491	6·36	1	70·96
43	6·57	497	6·55	1	75·65
36	7·16	459	5·03	1	64·10
42	7·65	500	5·49	1	65·36
43	8·46	475	5·14	1	56·82
49	7·15	466	6·85	1	65·18

B. *Milk.*

Amounts g. per 100 cc. milk			Ratios		
Lactose	Protein-N	NaCl	Lactose	Protein-N	NaCl
4·87	0·51	0·17	9·55	1	0·33
4·84	0·55	0·17	8·80	1	0·31
5·98	0·38	0·13	15·73	1	0·34
4·88	0·36	0·16	13·55	1	0·44
4·63	0·55	0·15	8·42	1	0·27
4·88	0·45	0·15	10·90	1	0·32

Blood-glucose was determined by Hiller, Linder and Van Slyke's [1925] modification of the Hagedorn-Jensen method—true sugar being found in this way and not total reducing power. Blood-amino-nitrogen was determined by the Van Slyke manometric method [1929] and chloride by the thiocyanate procedure [Whitehorn, 1921].

Lactose was determined in the milk by the Shaffer-Hartmann iodimetric method [1920], nitrogen by the Kjeldahl method, and chloride by thiocyanate titration.

It will be seen that the ratios for blood, amino-nitrogen : sugar : sodium chloride are approximately 1 : 6 : 65, while the ratios for milk are approximately 1 : 11 : 0·35. It is evident therefore that milk cannot be considered

merely as a concentrated (and transformed) transudate of blood-plasma *minus* colloids, and that, in order to arrive at the typical ratios found in milk, it would be necessary to assume that certain of the constituents of the transudate are reabsorbed into the blood-stream. Such a process is considered to take place in the kidney [Cushny, 1926]. In the latter case, however, re-absorption of water and of certain solutes takes place during the passage of the glomerular filtrate along the renal tubules. This implies the existence of two complementary cell structures, one for filtration and one for re-absorption. The histological picture of the mammary gland presented by Martin [1919] shows, however, that the blood capillaries have direct access to the secreting epithelial cells and that no complementary structures akin to the renal glomeruli and tubules are present. The mammary gland is therefore not provided with any apparent means of securing specific re-absorption of part of the transudate.

That this criticism is a serious one may be shown from the following quantitative data. The sugar content of the blood of the lactating cow may be taken as roughly 40 mg. per 100 cc. The experimental animals used by us secrete per 12 hours about 10 litres of milk containing 450 g. of lactose. In order to furnish this amount of lactose there would have to be filtered from the blood in 12 hours $\frac{450{,}000 \times 100}{40 \times 1000} = 1125$ litres of transudate. Thus, on the filtration hypothesis, of this 1125 litres of transudate 10 litres would appear as milk, while the remaining 1115 litres would be re-absorbed into the blood-stream. This hardly appears to be a likely process.

In our view the nature of the transudation is best explained by the second hypothesis, namely, the selective absorption of milk precursors at the membranes separating the secreting cells from the blood supply. In this connection it might be observed that the existence of a renal threshold value for such a blood solute as glucose is explained by preferential absorption of this substance by the cells of the tubules. Further, the substances which are most easily absorbed by the mammary gland (glucose, amino-acids) are precisely those for which renal threshold values exist. We suggest therefore that the membranes separating the cells of the mammary gland from the blood-stream have similar properties to those separating the blood-stream from the renal tubules. Such a similarity would account for the free passage of amino-acids and glucose into the mammary gland and for the comparative impermeability to inorganic salts such as sodium chloride. The postulated preferential absorption would not appear so complex if it could be shown that the amino-acids and sugar were absorbed into the mammary gland in the same ratio as that in which they occurred in the blood. Blackwood [1932] has, in fact, provided positive evidence on this point. Further support for this suggested resemblance of transudation in the mammary gland to tubular resorption in the kidney is to be found in the behaviour of phloridzin. Phloridzin has the property of paralysing the re-absorptive power of the renal tubules for glucose. We should therefore expect that administration of phloridzin to the lactating animal would be followed by diminution in lactose secretion.

This has been noted by Paton and Cathcart [1911] and others.

We suggest therefore that the first stage in milk secretion is the separation from the blood of a transudate which contains the organic milk precursors (glucose and amino-acids) in the proportions in which they occur in the blood, and the inorganic milk constituents, for example sodium chloride, in much smaller relative concentration.

We may mention in this connection our previous finding [Blackwood and Stirling, 1932, 2] that inorganic phosphate is not absorbed by the mammary gland. Since, as has been pointed out above, the supposition that the transudate contains glucose in the same concentration as in blood does not appear likely, it follows that the transudate must be more concentrated with respect to glucose and amino-acids than is blood.

It is appreciated that this view of the transudation from the blood-stream to the mammary gland is attended by the difficulties common to all attempts to account for the passage of fluids across physiological membranes. For example, the theory first suggested by the work of Tamman [1896] and Starling [1899] to account for the filtration from the blood-stream to the glomerulus of the kidney, and later developed by Govaerts [1923] and Schade and Claussen [1924] as a general theory of transudation across cell membranes considers the effective filtration pressure to be the hydrostatic pressure of the blood less the osmotic pressure of the plasma-colloids (oncotic pressure). If the membranes of the mammary gland are specifically permeable towards blood-solutes, then those blood-solutes towards which they are relatively impermeable will exert a high partial osmotic pressure in opposition to transudation. If we accept 150 mm. mercury as the typical arterial pressure and 35 mm. mercury as the typical oncotic pressure of blood, we obtain 115 mm. mercury as the effective filtration pressure. According to our observations, the partial osmotic pressure of sodium chloride in cow's blood is about 1·7 atmosphere or 1300 mm. mercury. It is difficult to see how transudation can be effected against such an oncotic pressure for sodium chloride. For the present, it must suffice to conclude that, in the mechanism of filtration across physiological membranes, considerations exist regarding the nature of which it is not at present justifiable to speculate.

Intracellular osmotic changes in the mammary gland.

Turning now to consider the nature of the osmotic changes produced in the transudate in the gland cells, it is of primary importance to recognise that, no matter what be the osmotic pressure of the transudate from the blood-stream to the gland, the onset of synthetic activity within the secreting cells will be accompanied by a lowering of osmotic pressure. The formation of one molecule of lactose from two molecules of glucose and the elaboration of osmotically inactive colloidal protein from osmotically active amino-acids are reactions involving lowering of osmotic pressure. The formation of insoluble colloidal calcium phosphate would have a similar tendency, but the

direction of the osmotic change involved in fat secretion is more difficult to judge. The precursors of milk-fat are probably of the nature of phosphatides and as these exist in the plasma in the colloidal state they will contribute but slightly to the osmotic pressure of the blood. If they are absorbed into the gland cells in a colloidal state, and if the reactions by which fat is formed take place out of true solution the secretion of fat will involve very little alteration in tonicity within the secreting cells. It is known that the fat content of milk is readily affected by external factors which have no influence on the concentrations of lactose and protein in the milk secreted. The synthesis of these latter constituents involves osmotic changes and their secretion will be controlled by osmotic relationships. The variability of the fat content of milk seems, accordingly, to indicate that fat secretion is unconcerned with the osmotic conditions in the mammary gland. It seems feasible therefore to consider that by far the largest part of any osmotic change taking place during milk secretion is to be accounted for by the synthesis of protein and carbohydrate.

Since milk is isotonic with blood, we have to consider how this lowering of osmotic pressure due to synthesis is related to the osmotic pressure finally attained during secretion—namely the osmotic pressure of milk. It will be apparent that the osmotic pressure attained at the end of synthesis will be a function of the amounts of glucose and amino-acids. It seems possible therefore to obtain data upon which to consider the osmotic relationships of the mammary gland by determining the freezing-point, lactose and protein contents of the milk secreted. In order to obtain such data we have collected and weighed the daily secretion of milk from six animals. The freezing-point of the milk has been determined by the Hortvet cryoscopic method [Hortvet, 1921] together with the amounts of water (by evaporation), lactose [Shaffer and Hartmann, 1920] and protein (Kjeldahl) secreted in 12 hours. These are shown in Table II. With the data available we propose to consider the nature

Table II.

Cow No. ...	1	2	3	4	5	6
Total yield of milk (g.)	8164	8164	9979	9298	10433	10866
Water (P) (g.)	7202	7129	8734	8174	9138	9429
Lactose (a) (g.)	389	387	586	443	471	519
Protein (b) (g.)	260	281	240	211	355	303
Depression of freezing-point (Δ)	0·531°	0·545°	0·544°	0·546°	0·545°	0·543°

of the osmotic changes involved in a hypothetical process the reverse of synthesis—namely the transformation of lactose back into glucose and protein into amino-acids. By this means it is possible to calculate the theoretical composition of the transudate from which the milk is derived.

One gram molecule (342 g.) of lactose dissolved in 1000 g. of water will have an osmotic pressure of 22·4 atmospheres at N.T.P. This is represented by a depression of the freezing-point of 1·86°. If the lactose is now transformed

into glucose there will be an increase in the depression of the freezing-point of approximately 1·86°. The increase for a g. lactose in P g. water will be:

$$1{\cdot}86 \times \frac{1000}{P} \times \frac{a}{342} = \frac{5{\cdot}44\,a}{P}.$$

A similar expression may be developed for the transformation of protein into amino-acids. In column 1 of Table III are given the amounts of the

Table III.

	Yield from 100 g. caseinogen g.	Molecular weight	Yield from 100 g. caseinogen g.-mol.
Glycine	0·4	75·06	0·00533
Alanine	1·8	89·07	0·02020
Valine	7·9	117·10	0·06745
Leucine	9·7	131·11	0·07399
Aspartic acid	4·1	133·07	0·03080
Glutamic acid	21·8	147·08	0·14820
Hydroxyglutamic acid	10·5	163·08	0·06437
Serine	0·5	105·07	0·00476
Proline	8·0	115·08	0·06950
Hydroxyproline	0·2	131·08	0·00153
Phenylalanine	3·9	165·10	0·02417
Tyrosine	6·5	181·10	0·03589
Cystine	0·3	240·26	0·00125
Arginine	5·2	174·15	0·02985
Histidine	2·6	155·10	0·01670
Lysine	7·6	146·13	0·05200
Tryptophan	2·2	204·12	0·00108
Total	93·2	—	0·64707

various amino-acids to be obtained by hydrolysis of 100 g. of caseinogen. The figures have been taken from Mitchell and Hamilton [1929] and while they do not represent a quantitative recovery they may be taken as an approximation. From these have been calculated the g.-molecular yield of each amino-acid (column 2). It will be seen that the hydrolysis of 100 g. of caseinogen will increase the osmotically active concentration of any solution by approximately 0·647 g.-mol. For b g. of caseinogen dissolved in P g. of water the increase in depression of the freezing-point will therefore be

$$1{\cdot}86 \times \frac{1000}{P} \times \frac{0{\cdot}647\,b}{100} = \frac{12{\cdot}02\,b}{P}.$$

This expression may be used to give an approximate idea of the change in osmotic pressure caused by the hydrolysis of milk-protein.

If we have a solution of depression of the freezing-point Δ, and containing P g. of water, a g. of lactose and b g. of protein, the depression of the freezing-point (Δ_1) after hydrolysis of the lactose and protein will be given by the formula

$$\Delta_1 = \Delta + \frac{5{\cdot}44\,a}{P} + \frac{12{\cdot}02\,b}{P}.$$

This value Δ_1 has been calculated for each of the six milks the analyses of which are shown in Table II. The values for Δ_1 are given in column 6 of Table IV. We have now to consider the relationship of these calculated values to osmotic phenomena within the mammary gland during secretion.

Table IV.

Cow No.	P	Q	a	b	Δ	Δ_1	Δ_2	W Cals.
1	7202	17080	389	260	0·531°	1·259°	0·244°	−0·650
2	7129	17190	387	310	0·545°	1·314°	0·225°	−0·688
3	8734	19910	586	349	0·544°	1·240°	0·239°	−0·722
4	8174	17240	443	307	0·546°	1·152°	0·259°	−0·541
5	9138	21670	471	355	0·545°	1·292°	0·230°	−0·844
6	9429	19600	519	303	0·543°	1·129°	0·213°	−0·592

It will be appreciated that, at the end of the hypothetical hydrolysis considered above, the milk constituents are in the form in which they were obtained from the blood. In other words, by a process the reverse of that which must actually take place in the mammary gland, we have regained the transudate stage. The depression of the freezing-point (Δ_1), calculated for each milk, will be that which the transudate from the blood-stream must have in order that the osmotic pressure reached after synthesis has taken place may be that of milk. We may conclude that in order that the secretion at the end of the synthetic stage may be isotonic with blood the transudate must have an osmotic pressure of about three times that of blood. If the value is greater than this, then at the end of synthesis, water will have to be absorbed from the blood to give a product isotonic with blood; if less, then, before the milk can become isotonic with the blood, water will have to be returned to the blood. It has been suggested by Hammond [1913] that an osmotic flow of milk through the mammary gland might be produced by the breakdown of a glycoprotein precursor into lactose and milk-protein. If such a process were to take place it is certain that synthesis of glycoprotein must first take place with consequent lowering of osmotic pressure. A flow of water could result from hydrolysis of a glycoprotein only if the osmotic pressure reached at the end of the synthetic reactions were equal to or greater than that of blood. As has been noted above, this requires the separation of a transudate of osmotic pressure much higher than that of blood. When we remember the many mechanisms utilised by the living organism for the preservation of intracellular osmotic equilibrium, the separation of an intracellular fluid from the blood of higher osmotic pressure than the blood itself seems a very unlikely process.

In view of the isotonicity of blood and milk it appears to us a more profitable hypothesis to consider that at no point in mammary tissue does the osmotic pressure rise above that of blood. On this view the first stage in milk secretion is the charging of the gland cells with a transudate isotonic with blood. Synthesis will then commence and, in order to preserve osmotic equilibrium, water will be returned to the blood-stream. When synthesis is complete, the cells will be filled with milk isotonic with blood. On this view it is impossible to account for the discharge of milk into the alveoli by an osmotic effect. When, however, we remember the difficulties which have beset attempts to explain the continuous flow of other secretions by osmosis, it

seems advisable to ascribe this phenomenon, so far as milk secretion is concerned, to agencies not yet elucidated. It is then possible to test the likelihood of this suggested mechanism of milk secretion in the light of experimental data presented in this paper.

For example it is possible to calculate the amount of transudate required according to the hypothesis for the secretion of a known amount of milk. For this purpose we shall assume that in the gland cells the return of water to the blood takes place after completion of synthesis, whereas in practice the two processes will take place simultaneously. We have previously seen that hypothetical hydrolysis of the protein and lactose in milk gives a fluid of higher osmotic pressure than milk. Since the transudate is considered to be isotonic with blood, the amount of water which must be separated from transudate during milk secretion is identical with that which must be added to the hypothetical fluid to bring the osmotic pressure to the same value as that of the blood.

Let P = g. of water secreted in milk in time t.
Q = g. of water in transudate in time t.
Δ = Depression of freezing-point of the blood.
Δ_1 = Depression of freezing-point of the milk obtained after hydrolysis of protein and lactose.

Then $$\frac{Q}{P} = \frac{\Delta_1}{\Delta}, \quad Q = \frac{P\Delta_1}{\Delta}.$$

The values of Q for the six milks examined have been calculated and are given in column 2 of Table IV. It will be seen that, on the theory suggested above to describe the various phases of milk secretion, approximately 2/3 of the water obtained by the mammary gland in the form of the transudate will be returned to the blood during synthesis, the remaining third appearing in the milk. While the figures can only be taken as rough approximations, it cannot be said that the theory imposes on the mammary gland the necessity of dealing with excessive amounts of fluid. The theory does of course require that cell membranes shall have a permeability from the blood towards the gland different from the permeability in the opposite direction. This is an assumption which has to be made in describing many biological phenomena.

The energy requirements of the mammary gland.

In a previous paper [Blackwood and Stirling, 1932, 1] we have pointed out the error implicit in the assumption that all the glucose absorbed by the mammary gland is utilised for lactose synthesis. We have suggested that part may be utilised in providing energy and it seems desirable to consider the amount of energy which would be expended by the mammary gland according to the theory of milk secretion suggested in this paper.

Synthetic reactions are considered to be almost thermoneutral, but it is

as well to have some idea of the energy required for the daily synthetic requirements of the mammary gland.

$$\underset{1346{\cdot}4 \text{ Cals.}}{2C_6H_{12}O_6} = \underset{1351{\cdot}4 \text{ Cals.}}{C_{12}H_{22}O_{11}+H_2O}$$

Glucose — Lactose

From the above equation it will be seen that the heat of formation of 1 g.-mol. of lactose from 2 g.-mols. glucose is about 5 Cals. The experimental animals used by us secreted on an average about 1·5 g.-mols. of lactose per 12 hours, as shown in Table II. This will require the expenditure of about 7·5 Cals. which could be provided by the combustion of about 2 g. of glucose. The other synthetic reactions of the mammary gland will also involve the completion of secondary bonds (carbon to oxygen and nitrogen) and it is not to be expected that the energy required for the synthesis of protein and fat will be much in excess of that required for the synthesis of lactose. It is to be concluded therefore that the energy required for synthetic reactions by the mammary gland is so small as to be negligible.

It has been pointed out that the assumption of a transudate isotonic with blood requires the return of water to the blood-stream during synthesis for the preservation of osmotic equilibrium. For convenience of treatment we may consider synthesis to go to completion without separation of water and to be followed by return of water to the blood in amount sufficient to bring the osmotic pressure again to that of the blood. We should thus have a lowering of osmotic pressure due to synthesis and the lowest value obtained would be represented by a new depression of the freezing-point (Δ_2) given by the formula

$$\Delta_2 = \Delta - \frac{5{\cdot}44\,a}{Q} - \frac{12{\cdot}03\,b}{Q},$$

where a, b, Q and Δ have the same meanings as before. Values of Δ_2 have been calculated for the six milks examined and are given in Table IV.

It will be seen that, according to the view expressed in this paper, the secretion of milk involves, in 12 hours, the concentration of a fluid containing Q g. of water and of depression of the freezing-point Δ_2 to milk containing P g. of water and of depression of the freezing-point Δ. Since the process of concentration is considered to take place by passage of water from a solution of low osmotic pressure to one of higher osmotic pressure separated by a semi-permeable membrane it will not entail the expenditure of energy by the mammary gland. This fact is best illustrated by treating the matter formally according to the methods for evaluating osmotic work.

If p_1 = osmotic pressure of the solution to be concentrated,
p_2 = osmotic pressure of the blood,
V_1 = initial volume of solution,
V_2 = final volume of solution,
and W = work done in effecting concentration.

Then $$dW = (p_1 - p_2)\,dv$$

$$W = \int_{r_2}^{r_1} (p_1 - p_2)\,dv = \int_{r_2}^{r_1} p_1 dv - \int_{r_2}^{r_1} p_2\,dv.$$

Since p_2 remains constant throughout the process we may write

$$W = \int_{r_2}^{r_1} nRT dv - p_2 \int_{r_2}^{r_1} dv,$$

$$W = nRT \log_e \frac{V_1}{V_2} - p_2 (V_1 - V_2).$$

Instead of V_1 and V_2 we may write Q and P since these are the amounts of water (in g.) in the transudate and milk respectively. p_2, the osmotic pressure of the blood, is the same as that of milk and at body temperature (37°) is equal to $\frac{22{\cdot}4 \times 310\,\Delta}{1{\cdot}86 \times 273}$ atmospheres $= 13{\cdot}7\,\Delta$ atmospheres. $n = \frac{\Delta P}{1{\cdot}86}$.

$$W = \frac{\Delta PRT}{1{\cdot}86 \times 1000} \log_e \frac{Q}{P} - \frac{13{\cdot}7\,\Delta}{1000}(Q - P) \text{ litre atmospheres}$$

$$= \frac{2{\cdot}303 \times 0{\cdot}082 \times 310}{1860} \Delta P \log_{10} \frac{Q}{P} - \frac{13{\cdot}7\,\Delta}{1000}(Q - P) \text{ litre atmospheres}$$

$$= 0{\cdot}03148\,\Delta P \log \frac{Q}{P} - 0{\cdot}0137\,\Delta\,(Q - P) \text{ litre atmospheres}$$

$$= 0{\cdot}000762\,\Delta P \log \frac{Q}{P} - 0{\cdot}000332\,\Delta\,(Q - P) \text{ Cals.}$$

This expression has been evaluated for each of the milks for which analytical data are available, the values of W so calculated being given in Table IV, column 8. In each case a negative value is obtained. In this fact we have a mathematical expression of the statement made above that the theory of the secretion of milk presented does not require the expenditure of any energy other than that required for synthesis.

It should be noted that when an energy balance is struck according to the equation:

Total energy requirements = energy for synthesis + energy for concentration,

the negative values obtained for the last factor are insufficient to give the total energy factor the negative sign. The conceptions are therefore in harmony with the idea of secretion requiring the expenditure of a small amount of work by the gland cells.

Summary.

The absorption of milk precursors by the mammary gland and the mechanism of milk secretion are considered from the physico-chemical standpoint.

The nature of the transudation from the blood-stream to the mammary gland may best be explained by the hypothesis of selective absorption of milk precursors at the membranes separating the secreting cells from the blood supply.

It is considered that during milk secretion a transudate is separated from the blood having the same osmotic pressure as blood, and that during synthesis water is returned to the blood to maintain the tonicity of the cell fluid. At

the end of synthesis the gland cells will therefore be filled with milk isotonic with blood.

Quantitative aspects of this theory of the mechanism of milk secretion are considered in the light of analytical data for the composition and yield of milk from six cows.

It is shown that this theory of milk secretion does not require the expenditure of any energy by the gland cells other than that necessary for the synthesis of milk constituents.

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CXXXIV. ON THE FUNDAMENTAL NATURE OF PARATHORMONE ACTION.

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In a preliminary paper [McGowan, Cunningham and Auchinachie, 1931], certain tentative conclusions were drawn regarding the intrinsic and essential nature of vitamin D action. A series of complete calcium and phosphorus balance experiments[1] on rabbits has since been carried out with the result that these provisional conclusions have been substantiated in full. The detailed description of these observations will be published in a subsequent paper. The chief deduction drawn at the time was that vitamin D produced its effect by breaking up the lipoids of the tissues and setting free phosphoric acid which was neutralised *in situ* by the calcium of the blood, thus bringing about local deposition of tricalcium phosphate. It should be noted that such precipitation is extra-vascular. A precipitation of calcium salts in various parts of the body following the injection of parathormone has been observed by various investigators [*e.g.* Taylor *et al.* 1931], and it seemed of interest to try to locate the exact site of this deposition. It was thought that this might throw some light on the mode of action of parathormone as also on the vexed question as to whether vitamin D acts primarily and solely through the intermediation of the parathyroid gland.

Because of an acquired familiarity with the histology of their organs, fowls were employed in the investigation: while, on account of their insusceptibility to parathormone [Taylor *et al.* 1931], large doses were administered daily to laying hens, the blood-serum of which has already, as is well known, a high calcium content. It was hoped by these means to obtain a definite result even in such a refractory animal.

The parathormone employed was prepared by Eli Lilly and Co., Indianapolis, U.S.A., according to Collip's formula and contained 20 units per cc. It was injected into the pectoral muscles. The fowls were kept in individual cages. They were fed *ad lib.* on a mash consisting of bran 2 parts, sharps 4 parts, maize 2 parts, crushed oats 1 part and soya bean meal $1\frac{1}{2}$ parts, to $10\frac{1}{2}$ cwts. of which mixture had been added 40 lbs. of a mineral mixture. They received also, *ad lib.*, a grain mixture consisting of equal parts of maize, wheat and oats: oyster shell grit and water were freely available.

[1] The analyses were carried out by Messrs William Godden, William H. Williams and Miss B. W. Simpson.

The calcium content of the blood-serum and the phosphorus content of the whole blood were determined from time to time[1], while, at the death of the fowls, sections were prepared from practically every organ and tissue of the body and treated by von Kossa's silver nitrate method for the detection of tricalcium phosphate.

A brief résumé of the findings in the various experimental fowls will now be given. The hens employed were all 2 year old laying White Leghorns.

Hen 611, brought into the cage from free range on 18. iv. 32: breast-bone quite straight, regular, thin and rigid[2]: no eggs had been laid by 20. iv. 32, when the calcium of the blood-serum was found to be 28·1 mg./100 cc. and the phosphorus of the whole blood 6·04 mg./100 cc.: 50 units parathormone were injected on this date and again next day when the fowl was seen to be rather dull. On 22. iv. 32 it was found to be nearly dead but blood was obtained which contained calcium 20 mg./100 cc. and phosphorus 12·8 mg./100 cc. On *post mortem* examination, the most notable features observed were the presence of small white patches of various sizes in the substance of the breast-muscles (the other muscles were not examined), the smearing of the whole surface of the peritoneal cavity with egg-yolk from a burst ripe ovum, and ovary and oviduct in full laying condition.

The nature of the white spots in the muscles is difficult to establish. In the patches, the muscle fibres were markedly swollen and showed considerable disintegration and necrosis. This subject will be touched on again later. As regards the egg-yolk in the peritoneal cavity, the most feasible explanation of its occurrence is that the egg, having burst from its follicle and having missed the oviduct, in its unprotected state is easily ruptured by the movements of the gizzard and intestine *etc.* There is no evidence to suggest that the bursting is brought about primarily by the parathormone, though it seems to have something to do with the failure to get into the oviduct.

The femur and tibia were very sclerosed and contained a very small amount of a pale marrow (hen originally was slightly anaemic). It may be stated here that in none of the experimental hens employed was there any evidence of osteoporosis or any change, atrophy or hypertrophy, of the parathyroids subsequent to injection[3]. The most important finding, however, was discovered microscopically on examining the sections which had been treated with silver nitrate. Deposits of tricalcium phosphate were found intravascularly in the following situations: capillaries of the lungs, especially at the periphery of the lobules near the chest walls, large veins of the liver, *artérioles à housse* of the spleen, large veins and arteries of the spleen (also in the *vasa vasorum* of these arteries) and the capillaries of the glomeruli of the kidneys (see Plate IV).

The distribution of the condition just described was very irregular in individual organs; and while in this fowl all the organs just mentioned were affected, in others of the series certain of these organs escaped. In every case, however, the lungs were involved. Unfortunately, the intestines of this bird were not examined for calcium deposition. The aorta was quite healthy. All the tricalcium phosphate precipitation found was intravascular with the exception of a trace in the tubules of the kidney and a slight occurrence in phagocytes in the periportal spaces of the liver.

Hen 621, brought into the cage from free range on 18. iv. 32: breast-bone normal: laid an egg on 19. iv. 32; and a shell-less one on 20. iv. 32: on this date the serum-calcium was 22·3

[1] These estimations were carried out by Dr D. W. Auchinachie and Mr A. R. G. Emslie.

[2] The condition of this bone has been regularly employed for some time as an index of the state of the skeletal system generally and has been found to be of very great assistance in this regard.

[3] One has observed fairly frequently cases of laying hens on free range with marked osteoporosis and great hypertrophy and greatly increased activity of the parathyroids together with the production of shell-less eggs. As such fowls were receiving large quantities of calcium in their food together with calcium grit, the condition would seem to be due to a lack of absorption of calcium brought about possibly by too low acidity of the intestinal tract.

mg./100 cc. and blood-phosphorus 3·19 mg./100 cc.: she laid a shell-less egg on 21. iii. 32: 50 units parathormone were given on 20th, 21st, 22nd and 23rd. She looked somewhat dull on the 23rd and was found newly dead, at 9 a.m. on the 24th: blood taken at once from the heart contained 14·6 mg./100 cc. calcium. The *post mortem* condition resembled that of hen 611, especially in regard to the white spots in the muscles, the smearing of the peritoneum with egg-yolk and the condition of the ovary and oviduct. There was no osteoporosis and no change in the parathyroids.

Hen 8, brought into the cage from free range on 22. iv. 32: breast-bone normal: serum-calcium 20 mg./100 cc. and blood-phosphorus 2·38 mg./100 cc. No eggs were laid during the experimental period. 50 units parathormone were given on 22. iv. 32 and again on 23. iv. 32; and 20 units on 24th, 25th and 26th, all at 9.30 a.m.: the hen was very dull on the 26th and was killed by bleeding at 2 p.m. The blood-serum contained 14 mg./100 cc. calcium and the whole blood 4·8 mg./100 cc. phosphorus. Again the findings *post mortem* were much as in the two previous cases but it was noticed that all the muscles of the body showed the white spots previously alluded to. There was no osteoporosis and no change in the parathyroids. Microscopically, the changes in the tissues were as in the previous two but less marked than in hen 611.

Hen 319, brought into the cage from free range on 25. iv. 32: breast-bone normal: blood-serum contained 27·3 mg./100 cc. calcium and whole blood 3·9 mg./100 cc. phosphorus: on 26. iv. 32 20 units parathormone; on 27. iv. 32 laid an egg and was given 20 units: on 28. iv. 32 laid an egg and was given 20 units; on 29. iv. 32 no egg; was given 20 units; on 30. iv. 32, 1, 2 and 3. v. 32 no egg, but 20 units each day; on 3. v. 32 serum-calcium 21·0 mg./100 cc. and blood-phosphorus 4·4 mg./100 cc.; on 4, 5 and 6. v. 32 no eggs laid, but 20 units each day at 9.30 a.m. killed by bleeding at 2.30 on 6. v. 32: serum-calcium 31·2 mg./100 cc. and blood-phosphorus 5·1 mg./100 cc. In this case there were no white spots in the muscles, no smearing of peritoneum with egg-yolk but the ovary was in full laying condition and there was an egg, complete with shell in the shell gland. Microscopically, tricalcium phosphate was found nowhere except in the capillaries of the lung. There was no osteoporosis and no change in the parathyroids.

Hen 566, brought into the cage from free range on 2. v. 32: breast-bone normal: an accident prevented the estimation of the serum-calcium but the blood-phosphorus was 6·2 mg./100 cc.: on 2. v. 32 50 units parathormone; on 3. v. 32 laid an egg and was given 50 units; on 4. v. 32 given 50 units; on 5. v. 32 laid an egg, given 50 units; on 6. v. 32 laid no egg, given 70 units at 9.30 a.m.: at 2 p.m. 6. v. 32 killed by bleeding: serum-calcium was 23·0 mg./100 cc. and blood-phosphorus 6·75 mg./100 cc. *Post mortem*, there were no white spots in the muscles, no egg-yolk on peritoneal surface and ovary and oviduct were in full laying condition; there was no osteoporosis and no change in the parathyroids. Microscopically, tricalcium phosphate was found only in the capillaries of the lungs.

It has been noticed that hens 611 and 621, which showed deposition changes so markedly, had greedily swallowed the calcium grit when put into the cages. It was thought that a resultant hypercalcaemia, produced in this way, might have had something to do with the results obtained and a test was carried out in this regard on hen 321.

Hen 321, brought into the cage from free range at 9.30 on 4. v. 32: breast-bone normal: fed at once by hand, a large quantity of oyster shell grit: blood-serum-calcium 29 mg./100 cc. and blood-phosphorus 5·8 mg./100 cc.: fed oyster shell grit again at 2 p.m.; and at 4.30 given 100 units parathormone into breast muscles: at 9.30 a.m. on 5. v. 32 the hen was very ill: it was killed by bleeding: serum-calcium was 18·8 mg./100 cc. and blood-phosphorus 17 mg./100 cc.: *post mortem* there were no white spots in the muscles but the peritoneal surface was smeared with egg-yolk, while the ovary and oviduct were in full laying condition. There was no osteoporosis and no change in parathyroids. Microscopically, deposition of calcium phosphate was found in the lungs and in a few tubules of the kidney. No definite answer to the question therefore was obtained but it has to be noted that the time between the first injection of the animal and its death was short. The experiment could not be repeated as the supply of parathormone was exhausted.

At this stage it was deduced from the observations just described that the parathormone was, in all probability, producing a condition of supersaturation of the blood with tricalcium phosphate and that this substance was separating out in the quieter parts of the circulation, as for instance in the large veins of the liver, while further it was being sifted out of the blood by the ordinary capillaries, wherever they formed a complex network, as for instance in the lungs, and

the glomeruli of the kidney, and also by the sinusoids of the liver and *artérioles à housse* of the spleen. A consideration of the illustrations seems to indicate that this conception was justified. The hypothesis was however put to the test by the intravenous injection into a fowl of a suspension of Chinese ink in saline solution, made by rubbing the solid ink up with the saline in a mortar and subsequent levigation to get rid of the larger particles.

Hen 544, was brought in from free range and injected intravenously by the wing vein with 35 cc. of such a suspension. During injection the comb became quite livid but later regained its normal red colour. The hen was killed 4 hours after injection, having in the meantime laid an egg: sections were prepared from practically every tissue of the body. Microscopically, carbon particles were found in the capillaries of the lung, the sinusoids of the liver and the *artérioles à housse* of the spleen[1] (see Plate IV). No carbon particles were seen in the large veins and arteries of the liver and spleen or in the glomeruli of the kidneys: on the other hand a considerable number were found in the phagocytic cells of the bone-marrow, the distribution being different in these respects from that of the tricalcium phosphate deposited in parathormone-treated hens.

The mucosa of various parts of the intestine of this hen was examined and carbon particles were found in fair quantity, not however in the capillaries but in the phagocytic cells of the tissue surrounding the capillaries in the villi. In this regard it was unfortunate that the mucosa of the intestines of such reacting hens as 611, 621 and 8 were not examined. The negative results obtained from examination of hens 319 and 566 cannot be regarded as conclusive because there was so little precipitation of tricalcium phosphate anywhere in these cases. The occurrence of the carbon particles in the phagocytes in this situation is very suggestive of a possible method whereby insoluble materials, such as iron refuse, tricalcium phosphate *etc.* may be removed from the body, the mobile phagocytes conveying them through between the epithelial cells on to the surface of the intestine.

The lung in this Chinese ink experiment provided another suggestive appearance, in that there were extravasations of blood into the air spaces (see Plate IV). The most feasible explanation of this was an embolic infarction by the carbon particles. The occurrence may serve to explain the extravasations of blood that have been found in the lungs and intestinal mucosa [Taylor *et al.* 1931] of highly susceptible animals, such as the dog, in parathormone and vitamin D poisoning[2].

The observations made in this investigation do not lend support to the idea that vitamin D produces its action primarily and essentially *via* the parathyroid glands. In considering this point, the relevant occurrences in vitamin D poisoning seem to be somewhat as follows. In the first place, a production of free phosphoric acid, as the primary effect of vitamin D action, appears to occur in the tissues which, in turn, attracts calcium from wherever it can, the intestine or bones, for neutralisation purposes. This need for neutralisation is urgent and calcium may be withdrawn from the blood more quickly than it can be replaced from the above sources. Hence a hypocalcaemia may develop which could serve to stimulate the parathyroid to activity, with the liberation of further quantities of calcium from the bones.

A pure parathyroid action could thus replace the original vitamin D action, if the process got out of hand. It should be noted, however, that, in this suggested mode of action, the parathyroid activity is secondary to the vitamin D activity (which can take place, as has been frequently shown, in

[1] The injection of Chinese ink intravenously provides an excellent method for demonstrating the *artérioles à housse* and for distinguishing them from the Malpighian arterioles.

[2] In the latter, although the main action is supposed to be in the tissues, accumulation of insoluble tricalcium phosphate in the blood-stream, as is to be suggested immediately in this paper, is not excluded. Large amounts of tricalcium phosphate are also frequently found in the intestinal mucosa in vitamin D poisoning.

animals deprived of their parathyroids). Further, parathyroid action differs intrinsically from vitamin D action in that it produces intravascular phenomena alone.

At present the most likely mode of action of parathormone seems to be a stimulation of the resorptive activities of the osteoclasts of the bones [*cf.* Hunter, 1930].

It seems possible that one may have been fortunate in these experiments in the selection of such an insusceptible animal as the fowl; for the smallness of the results produced has permitted of clearer observation and interpretation. A greater accumulation of tricalcium phosphate, in all probability, would have obscured the exact site of its deposition. The insusceptibility of the fowl is, in all probability, to be attributed to the fact that the laying hen tolerates with impunity serum-calcium concentrations as high as 25-30 mg./100 cc.

If hen 321, on account of the short duration of the experiment, be excluded from consideration, it will be seen that hens 611, 621 and 8, which showed tricalcium phosphate deposition and white patches in the muscle, also had their peritoneal surfaces smeared with egg-yolk from a burst ovum. This last phenomenon, on the other hand, was absent from hens 319 and 566 which showed tricalcium phosphate deposition in the lungs only and no white patches in the muscles. The following may be an explanation of these occurrences. It seems possible that the escape of the ripe ovum from the ovary may set in motion a train of events for the completion of the egg, one of which would be the provision in the blood of an extra supply of calcium for the formation of the egg-shell. Where this process is consummated by the ovum travelling down the oviduct, the extra calcium would be got rid of in this manner but the case would be quite other where the ovum was burst in the peritoneal cavity.

Taking into account the irregularity of occurrence and distribution of the calcium deposition as a whole in relation to similar occurrences in the muscles together with the actual histological appearances of the white spots, it seems not unlikely that these may be embolic infarcts due to occlusion of capillaries by deposited tricalcium phosphate. This question however is left open in the meantime.

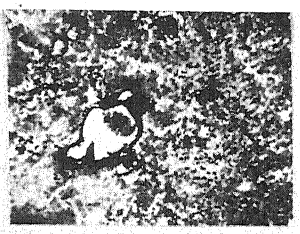
The calcium and phosphorus contents of the blood at various stages are recorded as found but no interpretation of them is attempted at present. It will be observed, however, that, in general, the blood-serum-calcium subsequent to several injections is lower than before injection.

Summary and conclusion.

The injection of large doses of parathormone intramuscularly into laying hens has produced a deposition of tricalcium phosphate inside the vessels as the main result. This, in all probability, is brought about by the parathormone liberating calcium hydrogen phosphate from the bones by a direct stimulation of the activity of the osteoclasts. This appears to be its only mode of



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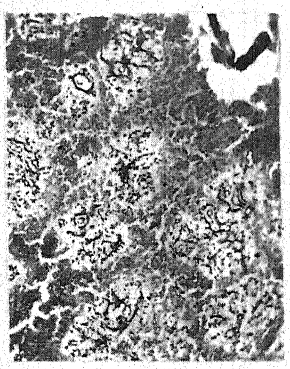
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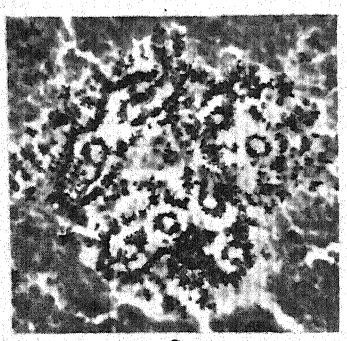
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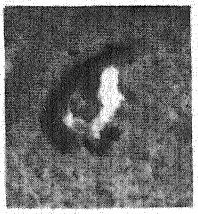
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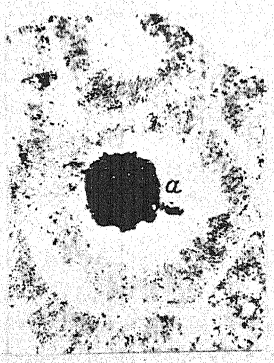
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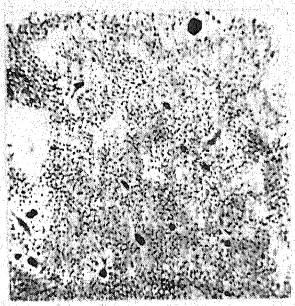
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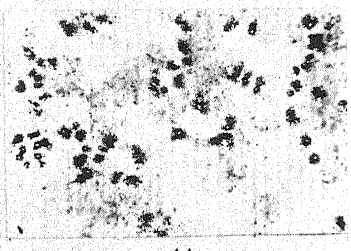
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action. Vitamin D however seems to be able to function in two ways, primarily, by liberating phosphoric acid in the tissues which attracts calcium from the intestine or bones by its acidity, and secondarily, by invoking the aid of the parathyroids through an induced hypocalcaemia.

The parathormone used in these experiments was supplied by Dr Clowes, Director, Eli Lilly Research Laboratories, to whom I wish to express thanks.

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DESCRIPTION OF FIGURES IN PLATE IV

Tricalcium phosphate appears deep black.

Fig. 1. Hen 8. Parathormone injection; lung; silver nitrate; low power; showing very large quantity of tricalcium phosphate in capillaries at periphery of lobules.

Fig. 2. Hen 611. Parathormone injection; liver; silver nitrate; medium power; showing large quantity of tricalcium phosphate in sinusoids and encrusting the walls of the veins.

Fig. 3. High power; vein in Fig. 2 showing the jagged incrustation of tricalcium phosphate. "*a*," blood clot.

Fig. 4. Hen 611. Parathormone injection; kidney; silver nitrate; high power; showing tricalcium phosphate in the capillaries of four glomeruli.

Fig. 5. Hen 611. Parathormone injection; spleen; silver nitrate; medium power; showing several Malpighian corpuscles with their *artérioles à housse* picked out by their tricalcium phosphate content; note absence of tricalcium phosphate from splenic pulp.

Fig. 6. High power of Malpighian corpuscle of Fig. 5; showing tricalcium phosphate in arterioles.

Fig. 7. Hen 621. Parathormone injection; spleen; silver nitrate; high power; showing artery with walls encrusted with tricalcium phosphate (section somewhat oblique).

Fig. 8. Hen 611. Showing vein (seen in Fig. 5) with walls encrusted with tricalcium phosphate; note jagged nature at places.

Fig. 9. Hen 544. Chinese ink injection; lung; unstained; low power; showing carbon particles in capillaries at periphery of lobules; also extravasation of blood into air space (*a*).

Fig. 10. Hen 544. Chinese ink injection; liver; unstained; low power; showing sinusoids filled with carbon particles.

Fig. 11. Hen 544. Chinese ink injection; spleen; unstained; low power showing several Malpighian corpuscles with their *artérioles à housse* picked out by their carbon content: also comparative absence of carbon from the splenic pulp.

(Figs. 9, 10 and 11 are much less highly magnified than the corresponding figures for the parathormone experiments, *viz.* Figs. 1, 2 and 5.)

CXXXV. THE DETERMINATION OF VITAMIN D IN THE LINE TEST BY MEASUREMENT.

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Introduction.

Shipley *et al.* [1921] showed that when cod-liver oil is administered to rats in which a rachitic condition has been induced by a deficient diet, calcium is deposited in the proliferative cartilage of the cartilage-shaft junctions of the long bones, forming a line across the bone at right angles to the longitudinal axis, the amount of the deposit being an indication of the extent to which healing has taken place. On the basis of this observation, McCollum *et al.* [1922] developed a technique for the determination of the ability of any agency to heal rickets, to which they gave the name "line test." In recording the results of tests carried out by this method, McCollum and his associates distinguished four grades of healing from + to + + + +, no healing being represented by a minus sign. Bills and McDonald [1926] later published coloured illustrations of stained bone sections typical of each healing grade. No special quantitative significance was attached to the differences in grade: they were adopted in the first place as a convenience for recording data. However, as data accumulated, Bills was able to establish a relationship between healing and dosage and he published [Bills *et al.*, 1931] a description of an improved technique of the "line" test by which the potency of a substance is estimated as the dose required to produce an average healing of + + in a large number of rats. From this is calculated a cod-liver oil coefficient, from the known dose of average cod-liver oil required to produce + + healing.

Coward [1928] had earlier introduced the use of a standard preparation of vitamin D with which the potencies of other substances could be compared, but this standard had not been adopted in the U.S.A. at the time of Bills's publication.

In the technique of the "line" test described by Dyer [1931] seven degrees of healing (from 0 to 6) are distinguished and the variation between average healing and dosage of the standard preparation has been determined. In the technique for the estimation of vitamin D by radiography described by Bourdillon *et al.* [1931] a similar procedure is followed, but the degree of healing is estimated from X-ray photographs instead of from stained sections, and 13 degrees of healing (0 to 12) are distinguished.

An essential feature of the recently described improved techniques is the estimation of the degree of healing by comparison with an ideal series, but in the method here described, the ideal series is dispensed with, and the degree of healing is expressed as the area of new calcification measured on magnified camera-lucida drawings of the bone sections. This has the advantages that a much finer gradation of healing is distinguished than is possible by the method of eye judgment, and that uncertainty in allotting the healing figure is eliminated. The error in matching the picture given by the test rat is considered by Bourdillon *et al.* to be responsible for one-third of the total probable error of the estimation carried out by their technique. In the technique of Bills *et al.* the difficulty involved in matching the picture against the ideal series is not so important, since only four grades of healing are distinguished.

Experimental.

The breeding stock are fed on a cooked diet consisting mainly of cereal offals with 10 % Trufood-brand skimmed milk powder, 5 % of Gromax and 2 % of red palm oil with 2·5 units of added vitamin D per g. In addition they receive about 10 cc. of milk daily and 10 g. of cabbage 3 times a week. On this diet, the bucks attain an average weight of 200 g. and the does an average weight of 150 g. in 95 days.

When rats are required for test, the mother of a litter is given the stock diet without milk, cabbage or the oil containing vitamin D from 1 week after the birth of the litter. The litters are not reduced in size, and we have not observed any objection to using litters of 10 to 13 rats for the test. The litter is weaned at 23 days, and if the rats are under 40 g. in weight, they are continued on the same diet as the mother had for a week. They are then transferred to the rickets-producing diet (Steenbock 2965), and maintained on it for 3 weeks to a month. An experimental period of 10 days is used. Doses of standard and other oil solutions are administered directly into the mouth from calibrated dropping tubes. Doses of margarine and butter are measured as cut lengths of ribbons obtained from calibrated glass syringes.

The day after the tenth dose has been given, the rats are killed by chloroform vapour, the distal ends of the radii and ulnae are dissected out, and left to harden in formalin overnight. A pair of bones is then sectioned and stained with silver nitrate. The stained sections are covered with water in a small porcelain dish which is placed on a platform attached to the upright of a Giltsch drawing stand. They are viewed through a Zeiss 8× aplanatic magnifier, and the drawings are made with the aid of an Abbé camera lucida. With the distance between the instrument and the drawing paper which has been selected, an overall magnification of 11 diameters is obtained.

The operations of sectioning, staining and drawing, require about 10 minutes per rat.

Measurement of the healing obtained.

When the initial rickets is very severe, the healing produced by a small dose of standard is usually shown by a dense black "line" with well defined edges, as shown in Fig. 1. The area of this "line" can easily be measured by a planimeter.

If the initial rickets is not very severe, or if the dose of vitamin D is large, the newly-calcified area is not completely separated from the shaft of the bone, but it can be distinguished by the pattern of the calcification, usually combining a dense black area with a number of radial lines, as shown in Fig. 2. The actual area of new calcification cannot then be measured so easily,

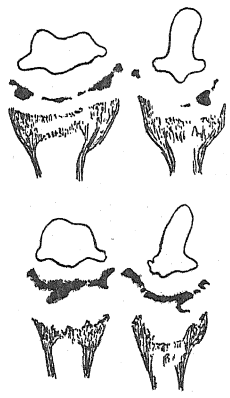


Fig. 1.

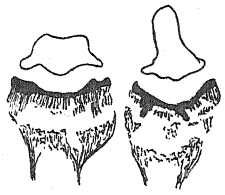


Fig. 2.

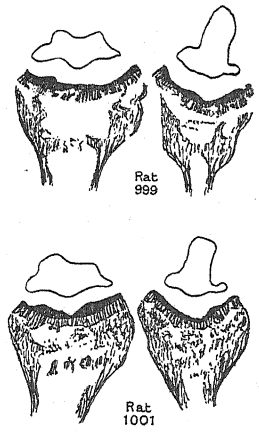


Fig. 3.

Fig. 1. Appearances of healing produced by small doses of vitamin D in rats with severe initial rickets. ×5·5.

Fig. 2. Appearance of healing produced by a moderately large dose of vitamin D. ×5·5.

Fig. 3. Litter-mates which received the same dose of vitamin D ($\frac{1}{2}$ unit). Though the general rachitic condition is more severe in rat 999 than in rat 1001, they show practically the same measured healing. ×5·5.

and it is our practice to measure the total densely calcified area *plus* half the area covered by scattered calcification. In the example given the area of dense calcification (on the original 11× magnified drawing) is 127 mm.^{2}, and the area of scattered calcification is 121 mm.^{2} The figure recorded is

$$127 + \frac{121}{2} = 187 \text{ mm.}^2$$

An illustration of the elimination of uncertainty in estimating healing produced is given in Fig. 3. The pictures reproduced are from litter-mates each receiving 0·5 unit of standard. On the basis of inspection one might place Rat 1001 in a higher grade than Rat 999, since the rachitic condition is less severe; but the measured areas of new calcification are practically equal. Though litter-mates, the two rats showed different degrees of severity of initial rickets.

It sometimes happens that the negative controls show some calcification similar in appearance to the new calcification of healing rickets. This may represent actual healing during the experimental period due to an error in feeding or due to loss of weight by the animals, or it may merely indicate that the initial rickets was not very severe because of a too generous supply of vitamin D in the mother's diet. In the last case the calculation of the assay is not affected (as will be shown below), but in calculating the general average values of the healing produced by doses of the standard preparation (plotted in Fig. 4) a correction has been made by simple subtraction when negative controls have shown such calcification. When the calcification represents healing produced by loss in weight, it is obviously exceptional and does not affect the remainder of the litter.

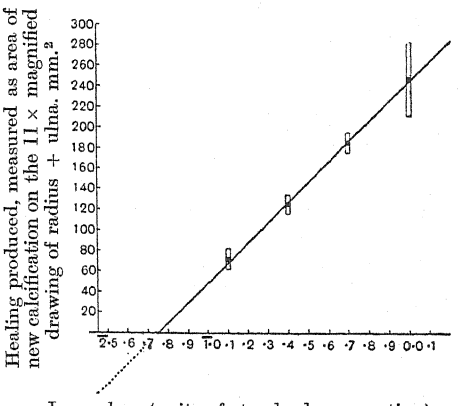


Fig. 4.

The relation between dosage and healing.

The values of the healing produced by different doses of standard in tests over a period of several months have been collected, and the average values are given in Table I. The value for 1 unit is based on the average of 6 rats only. For the three other dosage levels, the standard deviations were in fairly close agreement (29 to 34·5), and the probable errors have been calculated from the general value of the standard deviation determined for the whole series, which equals 33.

Table I.

No. of rats	Dose of standard preparation units	Average healing produced (mm.² on 11 × magnified drawing)	Probable error
44	0·125	72	3·4
58	0·25	125	2·9
43	0·50	185	3·4
6	1·00	247	12·2

In Fig. 4 the average values given in Table I are plotted against $\log_{10}$ dose of standard.

The unshaded area on either side of each point represents three times the probable error of the value. It is seen that the linear relationship fits the figures obtained within the limits of error.

The logarithmic relationship implies that doubling or halving the dose, whatever its value, will increase or reduce the measured healing by a constant

amount. This value has been determined by calculating the average value of the difference in healing between litter-mates receiving doses of vitamin D (whether of known value or unknown) in the ratio 2/1. The value obtained is 59·0 mm.^{2}, and the line given in Fig. 4 has the slope determined by this. The equation to the curve is

$$y = 196 \log x + 245$$

where y is the healing measured in mm.^{2} on the 11× magnified drawing of radius + ulna, and x is the dose of vitamin D in units. In interpreting the results of assays, the value of the constant 245 does not enter into the calculation, since the litters are always divided between the unknown substance and the standard preparation.

Another implication of the logarithmic relationship is the existence of a threshold dose which will just fail to produce any healing. This has frequently been confirmed in tests in which the lower of two doses in the ratio 2/1 fails to produce any healing or produces only a few mm.^{2}, while the higher dose produces healing of the order 60 mm.^{2} The average value of the threshold dose is given by the equation as 0·056 unit.

Interpretation of assays.

Assays may be interpreted by a general method for litters in which the doses are distributed in any way by reducing all the standards to one level and all doses of unknown substance to one level by calculation. The average values are then compared and an assay made for each litter. A weighted average of the separate litter assays is then taken as the final assay. This average must be calculated geometrically (*i.e. via* the logarithms of the values) since variations in doses corresponding to the normally variable healing will not be normally distributed.

Table II. *Assay of a sample of vitamin D concentrate.*

Litter	Rat	Dose	Area of new calcification mm.^{2}	Litter	Rat	Area of new calcification mm.^{2}
146	1103	Negative control	0	148	1118	0
	1110	,,	0		1119	0
	1102	¼ unit standard	92		1115	115
	1112	,,	88		—	—
	1113	,,	103		—	—
	1105	½ unit standard	198		1116	160
	1108	1 unit standard	243		—	—
	1101	$0{\cdot}125 \times 10^{-5}$ g. conc.	119		1117	131
	1107	,,	99		—	—
	1111	,,	127		—	—
	1104	$0{\cdot}25 \times 10^{-5}$ g. conc.	159		1114	147
	1109	,,	167		—	—
	1106	$0{\cdot}5 \times 10^{-5}$ g. conc.	167			

When the litters contain equal numbers of rats on comparable doses of the standard and the unknown, the animals may be considered in pairs and the differences between the individuals of each pair averaged. From this

difference the ratio of the doses may then be calculated by reference to the curve given in Fig. 4 or from the formula

$$(y_1 - y_2) = 196 \log \frac{x_1}{x_2}$$

where $(y_1 - y_2)$ is the difference in healing and x_1/x_2 is the ratio of the doses.

The calculations are illustrated by the assay in Table II.

Method I. (General method for any distribution of doses.)

The average healings produced by $\frac{1}{2}$ unit standard and by $0{\cdot}25 \times 10^{-5}$ g. of concentrate are calculated as follows.

Litter	Rat	Healing produced by $\frac{1}{2}$ unit standard	Rat	Healing produced by $0{\cdot}25 \times 10^{-5}$ g. concentrate
146	1102	92 + 59 = 151	1101	119 + 59 = 178
	1112	88 + 59 = 147	1107	99 + 59 = 158
	1113	103 + 59 = 162	1111	127 + 59 = 186
			1104	= 159
	1105	= 198	1109	= 167
	1108	243 − 59 = 184	1106	167 − 59 = 108
		Average = 168·4		Average = 159·3
148	1115	115 + 59 = 174	1117	131 + 59 = 190
	1116	= 160	1114	= 147
		Average = 167		Average = 168·5

From litter 146 the value of $0{\cdot}25 \times 10^{-5}$ g. of concentrate in terms of standard is given by

$$168{\cdot}4 - 159{\cdot}3 = 196 \log \frac{0{\cdot}5}{x},$$

$$x = 0{\cdot}5/1{\cdot}113$$

$$= 0{\cdot}45 \text{ unit.}$$

From litter 148 the value of $0{\cdot}25 \times 10^{-5}$ g. of concentrate in terms of standard is given by

$$168{\cdot}5 - 167 = 196 \log \frac{x}{0{\cdot}5},$$

$$x = 0{\cdot}5 \times 1{\cdot}017 = 0{\cdot}508 \text{ unit.}$$

The geometrical average of these two values, weighted for the number of rats in each litter (excluding controls) is 0·466 corresponding to

$$0{\cdot}466/(0{\cdot}25 \times 10^{-5}) = 186{,}000 \text{ units per g.}$$

in the original concentrate.

Method II. (Special method when litters are divided into pairs of rats.)

If one of the rats on $0{\cdot}25 \times 10^{-5}$ g. of concentrate had been omitted, the above two litters could have been considered as seven pairs on comparable doses of standard and unknown. Omitting rat 1104, the differences between the pairs are:

$$\begin{aligned} 92 - 119 &= -27 \\ 88 - 99 &= -11 \\ 103 - 127 &= -24 \\ 198 - 167 &= 31 \\ 243 - 167 &= 76 \\ 174 - 190 &= -16 \\ 160 - 147 &= 13 \\ \text{Average} &= 6{\cdot}0 \end{aligned}$$

This average difference corresponds to a dosage ratio of standard to unknown, in any of the pairs, of 1·073, *i.e.* $0{\cdot}5 \times 10^{-5}$ g. concentrate contains $1/1{\cdot}073 = 0{\cdot}932$ unit, or 1 g. contains 186,000 units.

Calculation of errors.

The errors to be expected in practice have been calculated by two of the methods described by Bourdillon *et al.* [1931]. The total number of rats available for consideration, however, is only about 200, and the experiments extend over a period of 7 months. The figures are, therefore, somewhat provisional.

The mean value for the increment obtained on doubling the dose is, as given above, 59·0 mm.^{2} The standard deviation of this value is 30·8 mm.^{2}, *i.e.* the probable error of any result calculated from n pairs of rats is $\frac{2}{3} \cdot \frac{30{\cdot}8}{\sqrt{n}}$.

In many litters several rats have received uniform doses of standard or unknown substance, or have been divided between two levels of dosage. When at least 4 rats have received the same dose, or can be reduced to the same level by calculation, the deviations from the mean for the litter have been calculated. The standard deviation calculated from these figures is 20·6. The standard deviation between two means is thus $20{\cdot}6 \times \sqrt{2} = 29{\cdot}2$, a value agreeing with the standard deviation for pairs of rats receiving doses in known ratio. It is of interest to note that the standard deviation from the mean for animals receiving the same dose is 20·6 if the animals considered are litter-mates, and 33 if the animals are not litter-mates (calculated from values for rats receiving the standard preparation over the whole period). Thus confining the comparisons to litter-mates reduces the probable error of the result by about one-third.

Taking 30 as the value of the standard deviation of the difference between two means, the errors to be expected in an experiment with n pairs of rats have been calculated.

Table III.

No. of pairs of rats	Percentage error corresponding to $\frac{2}{3}\frac{\sigma}{\sqrt{n}}$ "Probable error"		Percentage error corresponding to $\frac{2\sigma}{\sqrt{n}}$ 3 times "probable error"	
1	+27	−22	+102	−51
2	18	16	65	40
3	15	13	50	33
5	11	10	37	27
10	7·7	7·0	25	20
20	5·4	5·1	17	15
30	4·4	4·2	14	12
50	3·4	3·4	11	9·5
100	2·4	2·4	7·3	6·8

The values of percentage error corresponding to $\frac{2\sigma}{\sqrt{n}}$, *i.e.* the error likely to be reached or exceeded once in 22 groups, are plotted in Fig. 5, which shows

also the values calculated similarly by Bourdillon *et al.* [1931] for the estimation of vitamin D by radiography. It is observed that the errors now estimated are about two-thirds those given by Bourdillon *et al.*, which accords with their finding that about one-third of the probable error is due to error in the diagnosis of healing. With this reduction in the probable error, the number of rats required to produce an assay of a given degree of accuracy is approximately halved.

Sources of error.

Errors in measurement. The error in measuring the area of cross section of the new calcification deposit varies according to the type of picture presented. Small areas such as are shown in Fig. 1 can be measured to within a few mm.^{2}, but in measuring larger areas the error is more considerable. It is, however, not likely to be of importance when the healings produced in the animals compared are of approximately the same order. Assays based on a comparison of considerable healing produced by one substance with slight healing produced by the other are necessarily unsatisfactory, and can only be considered as approximate.

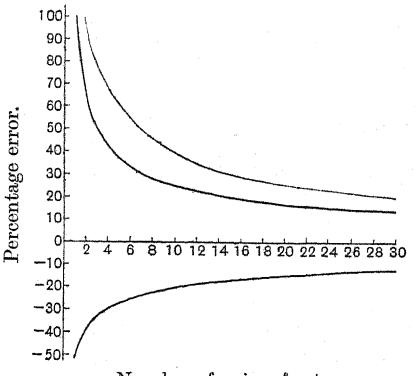


Fig. 5. Relation between error and number of pairs of rats in an assay. The curves show the percentage error corresponding to three times the probable error. The thin line is plotted from the values given for the estimation of vitamin D by radiography [Bourdillon *et al.*, 1931].

Slight errors in measurement may also be caused by the variation in the deposit at different planes of section and by slightly oblique viewing of the bone when making the drawing. These again will only affect to an appreciable extent the measurement of large areas.

Since the relation between healing and dosage is logarithmic, the errors in the assay from these causes are determined by the absolute errors in measurement and not the percentage errors. It therefore appears to be more satisfactory to select doses that produce slight healing ($\frac{1}{8}$ to $\frac{1}{4}$ unit). The variation in response in this neighbourhood does not appear to be greater than at higher levels of dosage.

The error in measurement could be reduced by using a higher magnification in making the drawing and by using both legs of each animal. The extra labour involved, however, does not appear to be justified by the slightly increased accuracy that would be obtained. It might, however, be possible to develop other more accurate methods of measuring the deposit, such as a photometric determination of the average blackness in the area of the line, or a micro-calcium estimation on the complete disc of newly calcified tissue dissected out from the remainder of the bone.

It may be mentioned here that in one routine assay the tibia was used as well as the radius + ulna. The calcium deposits were much less dense than in the radius + ulna, and were less suitable for accurate measurement.

Error due to variation in response. This error is responsible for nearly the whole of the error observed in practice. As is shown above, the error is much reduced by confining comparisons to litter-mates.

Other sources of error. The individual records in a number of vitamin D tests have been examined to determine the effect of the following variables: sex, weight of the animal at the beginning of the experiment, fore-period on the deficient diet, age and growth during the experimental period. None of these appears to exert any considerable influence. Growth during the experimental period appears to be inversely related to healing in many litters, but the effect is not uniform. The results are summarised below.

Influence of sex. The values of the healing produced by the standard preparation in 16 litters were considered. $\frac{1}{8}$-unit figures were converted to $\frac{1}{4}$-unit figures by adding 59, and $\frac{1}{2}$-unit figures were converted by subtracting 59. The healings for bucks and does in each litter were averaged separately and the litter averages were then averaged as follows: bucks 128 mm.^{2}, does 128 mm.^{2}

Influence of weight at the beginning of the experimental period. Considering the same figures, the weights of the rats and the healings were averaged irrespective of sex, and then the deviations from the mean healing for each litter were tabulated separately according to whether the individual exceeded or fell short of the litter average weight. For 30 rats exceeding the average weight, the average of the deviations was -1 mm.^{2} For 29 rats of less than the average weight, the average of the deviations was 0 mm.^{2}

Influence of the length of the fore-period. The values from 22 litters expressed as $\frac{1}{4}$-unit standard were averaged for each litter.

4 litters had a fore-period of 25/27 days,	mean healing	122	mm.^{2}
8 ,, ,,	less than 25 days, mean healing	126	,,
8 ,, ,,	more than 27 days, mean healing	128	,,

Influence of age at beginning of experimental period. 23 litters were considered. All were weaned at 23 or 24 days.

9 litters were less than 29 days from weaning,	mean healing	127	mm.^{2}
5 ,, 31 to 33 days from weaning,	mean healing	116	,,
9 ,, more than 33 days from weaning,	mean healing	129	,,

Influence of growth during the experimental period. The deviations from the mean healings for each litter were tabulated separately according to whether the individual's growth was more or less than the mean for the litter. Of 27 animals showing less than average growth, 16 showed more than average healing and 11 showed less. The average deviations were $-2{\cdot}25$ g. and $+2{\cdot}7$ mm.^{2} Of 27 animals showing more than average growth, 15 showed less than average healing and 12 showed more. The average deviations were $+2{\cdot}0$ g. and $-4{\cdot}9$ mm.^{2}

It is seen that, so far as the figures considered show, none of the variables exerts an appreciable effect, certainly nothing for which an allowance can be made. When a litter is being taken for test, pairs can be selected according to sex and weight, but it is impossible to pair animals according to the growth that they will make during the experimental period. Age and length of fore-period are the same for all the animals of one litter.

Variation in the healing produced by the standard preparation at different periods of the year.

The healings produced by different doses of the standard preparation in routine tests since September 1931, when the method was first applied, have been averaged throughout as $\frac{1}{2}$ unit. The results are summarised in Table IV.

Table IV.

Period covered	No. of litters represented	No. of rats	Mean healing as $\frac{1}{2}$ unit standard
18. ix. 31 to 23. x. 31	6	11	173
26. x. 31 to 18. xi. 31	7	19	186
21. xi. 31 to 21. xii. 31	6	26	187
21. xii. 31 to 6. i. 32	7	15	200
10. ii. 32 to 8. iii. 32	11	28	182
19. iii. 32 to 5. iv. 32	7	21	194
6. iv. 32 to 4. v. 32	10	23	167

During the corresponding period of an earlier year, Bourdillon *et al.* [1931] obtained a variation in healing produced by $\frac{2}{3}$ unit of standard, from about 6 scale divisions in September to 4·7 in February and 8·2 in April, corresponding to a spread of about 100 mm.^{2} in healing measured by the present method. It appears likely that the greater variation obtained is to be explained by a difference in the criterion of healing adopted. The present method does not take into account the general rachitic condition, but only the calcification in the line. As was shown in Fig. 3, two rats may show the same healing while showing general rachitic conditions of different degrees. So long as a line is obtained, the values are not likely to be affected by differences in the degree of severity of rickets developed in the animals before they are taken for test.

SUMMARY.

A method is described for the assessment of the degree of healing in the "line" test for vitamin D by measuring the area of new calcification shown on magnified camera-lucida drawings of the bone sections. In this way a much finer gradation of healing is distinguishable than is possible by the usual method of eye judgment, and uncertainty in estimating the degree of healing is eliminated.

Within the range studied, the healing measured thus is found to be proportional to the logarithm of the dose of vitamin D.

From an examination of the results so far available it appears that the expected probable error in an assay based on 10 pairs of rats is + 7·7 or − 7 %, and the error expected once in 22 tests is + 25 or − 20 %.

The chief source of error is the variable response of litter-mates to a given dose of vitamin. The influence of a number of variables such as sex and weight has been studied, and appears to be negligible.

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CXXXVI. THE ISOLATION FROM BILE OF A PIGMENT HAVING A DIRECT VAN DEN BERGH REACTION.

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THE pigments present in the blood-serum in jaundice were first differentiated by van den Bergh, who found that, whereas all icteric sera coupled with diazobenzene-*p*-sulphonic acid (Ehrlich's diazo-reagent) in the presence of alcohol, only certain of these sera would couple in simple aqueous solution. These diazo-reactions van den Bergh termed "indirect" and "direct" respectively, and it is now established beyond doubt that they are to be ascribed to two forms of bile pigment, the so-called indirect- and direct-reacting pigments. Subsequent work has shown that these pigments differ so much in other respects as to justify the conclusion that they are of distinctly different types; the evidence on this point has been summarised by Hunter [1930].

Careful investigation of the conditions under which bilirubin couples with diazo-reagent and comparison of solutions of this pigment in serum with indirect-reaction sera led us to the conclusion that bilirubin is responsible for the indirect reaction [Griffiths and Kaye, 1930, 1, 2], a view first put forward by Collinson and Fowweather [1926] and later by Roberts [1928] and Hunter [1930].

In regard to the nature of direct-reacting pigment, however, there is still much difference of opinion. The views of earlier workers were presented in our previous papers, so that we shall confine our attention to subsequent publications. Hunter [1930] found that if an alkaline solution of bilirubin is first buffered with disodium phosphate, it will couple with diazo-reagent in a distinctly acid medium. On the basis of this observation he suggests that direct-reacting pigment is sodium hydrogen bilirubinate, a view in accordance with that of Collinson and Fowweather [1926], who regarded the pigment as a salt of bilirubin, probably the ammonium salt. More recently, Fowweather [1932] has prepared the mono- and di-sodium salts of bilirubin, and these, when dissolved in serum, were found to give only delayed diazo-reactions. This finding seems definitely to exclude the hypothesis of Hunter [1930], and of Newman [1928], that the pigment is a sodium salt. In this connection it is of interest to recall that van den Bergh and Muller [1916] observed that a neutral solution of bilirubin would not couple with diazo-reagent. Fowweather was unable to demonstrate conclusively that ammonium bilirubinate gives a direct reaction.

Bilirubin is known to exist in more than one physical form, and Fowweather, after careful consideration of all aspects of the matter, has suggested that indirect-reacting pigment is a salt of another modification, more soluble in water, which tends to change into the indirect type. This author describes a pigment giving a direct reaction which is produced by precipitation of a solution of bilirubin in weak alkali, or alkali-phosphate, by a concentrated solution of a similar alkaline salt. This precipitate, when dissolved in serum, was found to give a typical prompt reaction, but on solution in water the product failed to react. The possibility that such a preparation might influence the reaction by virtue of its alkalinity must be considered in view of the fact that the behaviour of bilirubin with diazo-reagent is governed by the p_H of the medium in which the test is performed. Under the ordinary conditions, when equal parts of serum and reagent are mixed, the p_H of the mixture approximates to 2·4; slight variations in technique do not appreciably alter this value. The pigment which couples at this low p_H is of course the direct pigment; this pigment will in fact couple if the p_H lies below about 5·0. Bilirubin does not couple under these conditions, but it can be made to do so if the serum is so buffered, or made alkaline, that the final p_H after addition of the reagent is approximately 5–6. It seems therefore that the mere observation that coupling occurs in an acid medium is insufficient to exclude bilirubin unless the reaction of the medium is shown to be on the acid side of p_H 5·0. In fact, as Fowweather rightly points out, the observation that by addition of sufficient alkali an indirect-reacting serum can be made direct is valueless if, by such treatment, the serum is rendered more alkaline than direct-reacting serum. There can be no doubt that many products described in the literature as direct-reacting were, in point of fact, bilirubin giving a direct reaction because of the alkalinity of the medium in which it was dissolved. We found that when the p_H of serum containing bilirubin was raised to 10 by addition of NaOH, prompt coupling occurred [Griffiths and Kaye, 1930, 1], but for the reason already given such serum is not identical with direct-reacting serum. No artificially prepared serum showing a direct reaction can be considered as identical with the direct-reacting serum of jaundice unless it is shown to couple with diazo-reagent at $p_H < 2$.

Attention must, however, be called to an experiment carried out by Fowweather in which an ammoniacal solution of bilirubin was precipitated with saturated ammonium carbonate solution and the precipitate washed with a saturated solution of sodium chloride; when dissolved in serum the precipitate gave a prompt reaction. In this case most of the alkali must have been removed during the process of washing and further investigation of this interesting observation is clearly desirable.

On the whole, most workers have taken the view that direct-reacting pigment is either bilirubin which has undergone some intramolecular rearrangement, or that it is a salt or other derivative of bilirubin; the evidence which has been put forward in support of these conceptions is not, however, at all

convincing. Curiously enough, practically all the work which has been carried out on the direct-reacting pigment has been synthetic in character, although van den Bergh originally obtained the direct reaction with bile, in which, presumably, the pigment is present in considerable amount. Possibly the difficulties arising from the extreme instability of the pigment have blocked the analytical approach to the problem of its identity. Our knowledge of the physical properties of the pigments, both direct and indirect, and of icteric sera generally, has been much enriched by the many investigations, but it cannot be said that they have brought us anywhere near a solution of the chemical nature of direct-reacting pigment.

In our earlier work [Griffiths and Kaye, 1930, 1] we found that the direct-reacting pigment is present in dried gall-bladder bile after extraction of the bilirubin with chloroform, *i.e.* it is present in that fraction of the bile which is not soluble in chloroform. The preparations with which we were then working were crude, being contaminated by bile salts, *etc.*, but we were able to show that solutions of this material in serum were identical with the direct-reacting serum of jaundice. As a result of further work we have been successful in the isolation from this crude material of a pigment capable of giving a true direct reaction. Briefly, our procedure has been as follows: the chloroform-insoluble residue of dried bile was dissolved in weakly alkaline solution and precipitated by acetone. The direct-reacting pigment is adsorbed by the precipitate, from which it was obtained by extraction of the dried product with 80 % acetone. Evaporation of the acetone solution left a pigmented residue which gave a powerful colour with diazo-reagent at low p_H. The direct-reacting pigment was found to be precipitated from this acetone-product by addition of acids to its aqueous solution.

Experimental.

Human gall-bladder bile, obtained at autopsy, was used. Bile from this source was found to be very variable in its response to diazo-reagent; only those specimens which showed a good prompt direct reaction were employed. When necessary the bile was preserved for short periods in a well-stoppered bottle in a refrigerator from which light was excluded. Light has a very deleterious effect upon the direct-reacting pigment, and for this reason at all stages of the preparation the material was as far as possible preserved in a dark place.

Isolation of the pigment-bile salt complex.

The first stage in the extraction of the direct-reacting pigment was the isolation of a complex substance consisting of the pigment associated with the salt of a bile-acid. The gall-bladder bile was dried *in vacuo* over sulphuric acid and finely powdered. The powdered bile was treated with alcoholic HCl (conc. HCl, 1 cc.; alcohol, 100 cc.) until the mixture was acid to moist litmus paper. In our earlier work this mixture was made acid to Congo red, but

experience has shown that this degree of acidity is not only unnecessary, but distinctly harmful; direct-reacting pigment becomes increasingly unstable with diminishing p_H, and acidity strong enough to affect Congo red may often ruin the preparation. By experiment it was found that a p_H of 6·5–5·5, as determined by the glass electrode, was sufficient to ensure complete solubility of the bilirubin in chloroform without adversely affecting the direct-reacting pigment. After again drying *in vacuo* the finely powdered material was extracted with dry ether for 1–2 hours in a Soxhlet apparatus, using a 22 × 80 mm. thimble; in this way a good deal of pigmented fatty material was removed. Finally, to remove bilirubin, the material was extracted with dry chloroform in a modified Soxhlet. In the ordinary apparatus the chamber containing the thimble becomes considerably heated when using chloroform, with destructive effect upon the direct-reacting pigment; we were able to overcome this difficulty by slightly modifying the apparatus (Fig. 1). Extraction was continued until traces only of pigment were being taken up by the chloroform (6–9 hours). The chloroform was removed from the sticky residue *in vacuo*, and the material powdered. If it appeared that some parts of the substance had escaped extraction the process was repeated for a further period of time. The resulting yellowish-brown powder should give a prompt and marked reaction with diazo-reagent; if it fails in this it is useless to proceed and the specimen must be discarded.

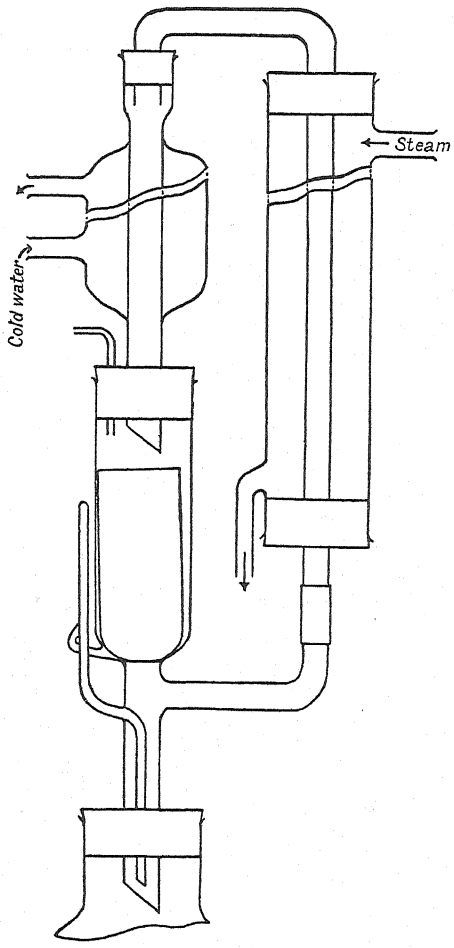


Fig. 1.

The product after extraction with chloroform consisted of the direct-reacting pigment admixed with bile salts, mucin, *etc.*; separation of the pigment from this material proved to be a matter of great difficulty. We found, however, that addition of acetone to an aqueous alkaline solution of the material resulted in the formation of a precipitate, on which, to a very large extent, the direct pigment was adsorbed. We therefore proceeded as follows. The powder was rubbed up with water in a mortar, using about 40 cc. for every 10 g. of material. The dark-brown viscid mixture was made alkaline to

litmus with a few drops of *N* NaOH, transferred to a flask, and precipitated by addition, with shaking, of four times its volume of acetone (distilled from $CaCl_2$). The mixture was shaken vigorously and filtered immediately. The precipitate was taken up in sufficient water to form a cream, and was re-precipitated with another 4 volumes of acetone. The precipitated material was filtered off, washed with acetone, and dried *in vacuo*. The filtrates from these operations, containing mainly bile salts and some pigment, were discarded. The dry acetone-precipitate had an amorphous, sometimes fibrous, appearance, and varied in colour from pale golden-brown to chocolate. It contained N, 7–8 % and P, 1–1·5 %, and was readily soluble in water yielding a solution having a powerful direct diazo-reaction. In the case of strong solutions the colour was masked by a precipitate produced by the reagent, but when this precipitate was filtered off, an intensely purple filtrate resulted, and much azo-pigment could be washed away with water from the residue on the filter-paper. It appeared from this that the substance was split by the reagent, probably because of the acidity of the latter. This was confirmed by the observation that an aqueous solution of the material was precipitated by the reagent acid alone. An equal volume of van den Bergh's solution A was added to a solution of the substance precipitated by acetone, and after being allowed to stand for some minutes, the mixture was filtered. Addition of reagent to the feebly coloured filtrate gave only a very weak reaction, whereas the precipitate reacted strongly with the reagent. The precipitate rapidly lost its reactivity on being left exposed to the air.

The material precipitated by acetone consisted in the main of pigment and mucin. By experiment it was found that the pigment was best extracted from the dry powder by cold 80 % acetone. The material was finely powdered, rubbed up with successive quantities of about 20 cc. of 80 % acetone (80 cc. anhydrous acetone made up to 100 cc. with water) and allowed to stand for 5–10 minutes after each treatment, when the supernatant fluid was decanted on to a filter. After about 10 washings in this manner very little pigment was taken up by the acetone. This operation was carried out by artificial light. Without delay, the acetone was removed from the dark reddish-brown filtrate under reduced pressure in an atmosphere of coal-gas, the containing flask and its contents being warmed to about 60°. When, on account of frothing, further distillation was impossible, any precipitated fatty material was filtered off and the residue evaporated to dryness over sulphuric acid *in vacuo*. The yield, which was variable, amounted to 1–3 % of the chloroform-extracted bile. This preparation will be referred to as the acetone-product.

The nature of the acetone-product.

The dry residue was a reddish-brown, scaly, amorphous and hygroscopic substance, extremely soluble in water, moderately so in alcohol; when powdered it appeared bright orange in colour. Heated on platinum foil, the substance left a very small infusible alkaline ash. Its aqueous solution was

lemon-yellow or orange-red according to concentration, and gave Hay's test for bile-acids. Solutions in normal serum gave an excellent prompt direct diazo-reaction, and were strictly comparable with direct-reacting serum. The intensity of the diazo-reaction of this product was most striking; weak aqueous solutions, containing as little as 1–2 mg./100 cc., gave an immediate purple colour, at approximately p_H 1·5, on addition of an equal volume of reagent. With more concentrated solutions the diazo-reagent produced a precipitate which obscured the colour of the azo-compound, but filtration resulted in an intensely purple filtrate. If allowed to stand for some time after addition of the reagent and then filtered, a small green residue remained on the filter-paper; this was considered to correspond to the oxidation product already described [Griffiths and Kaye, 1930, 1].

The product exhibited marked instability, especially when in the acid state; the pigment thrown down by acids rapidly became green and lost its power to react with diazo-reagent. Aqueous solutions, which at first were lemon-yellow in colour, rapidly became decolorised on exposure to light and ceased to give a diazo-reaction; preservation in a dark place greatly delayed this change.

Acids generally caused precipitation of strong solutions of the product in water when the p_H was brought below about 3·0, whereby practically all the pigment was removed from solution. The precipitated pigment was soluble in alkalis and contained nitrogen and traces of phosphorus.

As it appeared that the pigment represented a small fraction only of the acetone-product, experiments were made in order to determine the nature of the rest of the material. A fairly strong solution of the acetone-product in water was treated with dilute HCl until precipitation was complete, and after filtering NaOH was added to the filtrate until it was only faintly acid to litmus. The neutralised fluid was evaporated to dryness on a water-bath. A pinkish-white, hygroscopic residue was obtained, readily soluble in water to yield a solution which gave Hay's and Pettenkofer's tests; much of the substance was therefore a bile salt. Microscopically the residue, apart from sodium chloride, appeared to consist only of colourless crystals of roughly hexagonal shape.

The relationship between the pigment and this bile salt was not further investigated at this stage, but it seems probable that the association of these bodies was merely accidental, although it is remarkable that, whereas the greater part of the bile salts is removed during the initial precipitation of the bile-residue with acetone, this fraction constantly escaped elimination. The action of acids, which presumably decompose an alkaline salt of the direct-reacting pigment with resulting precipitation of the pigment, leaving the bile-acid in solution, appeared to be reversible; when by addition of alkali the change was reversed, the pigment re-dissolved, and on evaporation to dryness *in vacuo* no significant alteration in the character of the product could be detected.

Isolation of the direct-reacting pigment.

As the pigment appeared to be readily separated by addition of weak acids to its aqueous solution, this method was adopted for its isolation. Care was necessary, however, in view of the harmful influence of strong acidity, and acetic acid was at first employed. The powdered material was treated with glacial, or with alcoholic, acetic acid, dried *in vacuo*, and the dried material extracted with chloroform in order to remove any traces of bilirubin. The amount of chloroform-soluble material was found to vary considerably in different preparations; some showed practically none, others a fair amount. Although some of this pigment was undoubtedly bilirubin, often much was the direct-reacting pigment, whose solubility in chloroform seemed to vary from preparation to preparation. After extraction with chloroform the products, when dissolved in weak alkali, were highly reactive towards the diazo-reagent, giving a true direct reaction. The material varied in colour from orange-red to dark greenish-brown, probably because of the presence of some oxidation products; total nitrogen (micro-Kjeldahl) estimations on six different samples gave values ranging from 3·7 to 5·5 %, and the preparations could not, therefore, be regarded as of uniform composition.

Better results were obtained by precipitation of an alkaline solution of the acetone-product with dilute mineral acid. The acetone-product readily dissolved in water to give a deep orange-red solution of p_H 7–8. Addition of $N/10$ HCl dropwise resulted in a gradual change of colour to a more greenish hue, and ultimately to definite greenish turbidity or actual precipitation at p_H 3·6–4·0. When precipitation did not occur it was readily induced by addition of a little solid sodium chloride to the solution. The greenish pigment, which appeared reddish-brown by transmitted light, was centrifuged, and the supernatant yellowish-green fluid removed. The precipitate was dissolved in $N/100$ NaOH, and the golden-brown solution treated with just enough $N/10$ HCl to re-precipitate the pigment. It was observed that the pigment separated at the same p_H as in the first case; it is interesting to note that bilirubin is precipitated from its alkaline solutions at p_H 6·8. The precipitate was centrifuged, washed 2 or 3 times with $N/100$ HCl, and dried *in vacuo*.

The product was a greenish amorphous powder, practically insoluble in water, but readily soluble in alkalis and in alcohol, in which it yielded a greenish-yellow solution, which rapidly darkened on standing. Solubility in alcohol was not always complete. In chloroform the solubility was variable, some samples showing practically none, others a fair amount; it was noticed that it was particularly those samples showing incomplete solubility in alcohol which also showed chloroform-solubility, which was thought to be due to bilirubin. Bilirubin being practically insoluble in alcohol, the pigment was treated with this solvent and filtered; a small brown residue on the filter-paper was found to be soluble in chloroform. After evaporation of the alcohol *in vacuo*, the dark-green amorphous residue was practically insoluble in water

and in chloroform, but readily dissolved in alkalis to yield a golden- or reddish-brown solution. When dissolved in a little $N/100$ NaOH, the solution, after dilution with water, gave an immediate and strong reaction with diazo-reagent at p_H as low as 1–2. In this connection we must remark the very unstable nature of the pigment, especially in the presence of free acid; although the majority of the products of the action of acid on the acetone-product gave a good van den Bergh reaction, occasionally a product was obtained which did not appear to react promptly. In such cases it was observed that the reaction mixture was clouded by a fine green precipitate; when this was filtered off, the filtrate was found to be highly coloured by the azo-compound. The green material, the amount of which increased with the age of the specimen when it was exposed to air and light, is an oxidation product, and is not reactive towards the diazo-reagent. The pigment contained no phosphorus, evolved pyrrole when distilled with zinc dust, and rapidly lost its power to give a diazo-reaction on exposure to air and light.

The following analytical data were obtained for preparations from different samples of bile (mol. wt. by Rast's method, N by micro-Kjeldahl): (*a*) M.P. 145–150°, mol. wt. 721, N, 4·6 %; (*b*) M.P. 142–150°, mol. wt. 688, N, 3·9 %; (*c*) M.P. 145–150°, mol. wt. 696, N, 4·5 %; (*d*), (*e*), (*f*) and (*g*) N, 3·4, 4·3, 4·3, 4·7 % respectively. The probable error in the nitrogen determinations is large on account of the small amounts of substance available for analysis. Micro-analysis of samples (*a*) and (*c*) showed: (*a*) C, 60·4; H, 7·71; N, 4·35 %; (*c*) C, 64·0; H, 8·48 %; $C_{32}H_{50}O_{11}N_2$ requires C, 60·2; H, 7·89; N, 4·39 %. Further investigation at this stage was precluded by the small amounts of material available. It is obvious that there are discrepancies in these results greater than the probable experimental error, which is not surprising in view of the instability of the pigment, the small amounts obtained, and the fact that some degree of oxidation is unavoidable.

In the absence of more precise data, which must await the investigation of larger quantities of the product, the present results indicate the general nature of the pigment; we feel that there is ample justification for supposing that we were, in fact, dealing with a chemical entity. The low N content is of interest; the N in the samples analysed averaged 4·3 % whereas bilirubin ($C_{33}H_{36}O_6N_4$) contains 9·6 %; this finding alone shows that we were dealing with something other than a modified form of bilirubin. So far as we are aware, there is one other blood-pigment derivative only which is known definitely to contain two N atoms in the molecule, namely, bilirubinic acid ($C_{17}H_{24}O_3N_2$), a reduction product of bilirubin [Fischer and Röse, 1912]. Bilirubin and related derivatives of haemoglobin which have been sufficiently studied contain four N atoms, probably in the form of four pyrrole residues, but in the case of bilirubinic acid two pyrrole groups only remain attached to one another. It seems not unlikely, therefore, that the direct-reacting pigment is similar in nature to bilirubinic acid.

The pigments present in the blood-serum in jaundice have come to be

named after the type of van den Bergh reaction which they give, *i.e.* direct- and indirect-reacting pigments; these terms are clumsy and do not fit in with the nomenclature of the bile pigments. Harrison [1930] has suggested that the pigments should be designated according to their origin in the body by addition of a prefix to the word bilirubin. Thus the indirect-reacting pigment, which is associated with jaundice of the haemolytic type, takes the name of *haemobilirubin*; direct-reacting pigment, which is formed from bilirubin in the liver and excreted along with the bile, Harrison calls *cholebilirubin*. These names are in every way suitable and should, we feel, be brought into common use.

SUMMARY.

The isolation from gall-bladder bile of a pigment having a direct van den Bergh reaction is described. This pigment, for which the name of cholebilirubin is recommended, is shown to exhibit those properties known to be associated with the direct-reacting pigment of obstructive jaundice. Cholebilirubin is not a modification of bilirubin, but a separate and distinct pigment with certain features in common with the other bile pigments; analysis, which must be regarded as preliminary in nature, is in favour of the formula $C_{32}H_{50}O_{11}N_2$.

I wish to express my gratitude to Prof. Hugh MacLean for his encouragement, and to the Medical Research Council for a grant. The micro-analyses were carried out by Dr Schoeller in Berlin.

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CXXXVII. ABSORPTION SPECTRA OF SUBSTANCES DERIVED FROM VITAMIN A.

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In continuation of earlier work on vitamin A we have investigated the ultra-violet absorption of certain products obtained after the vitamin has been decomposed. At the same time, we have tried to elucidate the mechanism of the antimony trichloride colour test.

As is well known, the blue colour is transient, and for purposes of assay it is necessary to determine the intensity of colour during the first minute, as otherwise the rapid fading tends to vitiate the results. Little is known concerning the secondary reactions which occur, and even the primary process is not fully understood. For the full development of the blue colour, the reagent must be nearly saturated and, it would appear, the $SbCl_3$/vitamin A ratio must be very high (order 20,000 : 1). This fact suggests that the blue substance (or substances) is an addition compound; the hypothesis of more than one coloured addition complex has already been advanced to account for the two apparently independent absorption bands seen in the blue solutions [Gillam and Morton, 1931; Heilbron, Gillam and Morton, 1931]. It is possible that the primary process, resulting in the blue colour, leaves the vitamin A molecule intact, whilst the secondary reactions associated with the fading of the colour bring about decomposition. To test this view, the blue solutions have been poured into large volumes of water so that the antimony is precipitated as oxychloride. The organic matter is then recovered and its properties studied.

The general method adopted is as follows. A known weight of liver oil or concentrate dissolved in chloroform is treated with a saturated solution of antimony trichloride in chloroform and the mixture shaken for one minute to allow the blue colour to develop fully. The solution is then poured into water in a separating funnel and shaken vigorously to complete the decomposition of the antimony trichloride. As it is impossible to separate the chloroform layer owing to the precipitated antimony oxychloride, the latter is brought into solution by careful addition of concentrated hydrochloric acid, when separation into two layers readily occurs. The chloroform extract is washed successively with sodium carbonate and water and dried over sodium sulphate. After removal of solvent, the residual oil (or concentrate) is very similar in appearance to the original material and in all cases gives a blue or

purple colour with antimony trichloride, similar to, but weaker than, that given by the original.

An examination of the ultra-violet absorption spectra of the solutions in chloroform reveals, however, as shown in Fig. 1, in place of the broad vitamin A

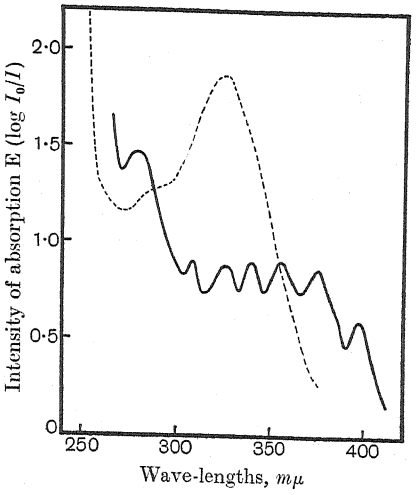


Fig. 1.

– – – – – Cod-liver oil 1 % (1 cm.) alcohol.
——— The same oil "regenerated" 1 % (1 cm.) chloroform.

Table I.

Material	Blue colour with antimony trichloride: $E^{1\%}_{1\,cm.}$	Wave-length max. mμ	$E^{1\%}_{1\,cm.}$	Wave-length max. mμ	Intensity of ultra-violet absorption $E^{1\%}_{1\,cm.}$ 328mμ gross value	Positions of maxima in the ultra-violet absorption spectrum (solutions in chloroform) wave-lengths of maxima in mμ
Cod-liver oil A	1·29	606	1·11	575	1·15	
Do. regenerated	0·96	615	0·77	575	0·72	—, 399, 376, 356, (340), (325), —, (279)
Cod-liver oil B	2·7	603	2·3	573	2·0	
Do. regenerated	1·16	616	1·05	580	1·1	—, 399, 377, 357, 342, 325, 308, (286)
Cod-liver oil C	2·5	604	1·92	572	1·88	
Do. regenerated	1·1	615	1·0	572	0·88	—, 398, 376, 357, 340, 324, 308, 280
Halibut oil	20·4	615	not examined		—	
Do. regenerated	4·6	613	not examined		4·75	—, 402, 378, 358, 340, (324), (309)
Old halibut non-sap.	66·5	616	55·4	583	58	
Do. regenerated	20·7	610	31·9	581	35	(423), 402, 377, 356, 340, (327), —, —
Cod-liver oil non-sap.	213	615	124	580	115	
Do. regenerated	—	no band	92	586	67	425, 400, 379, 357, (340), —, (310), (285)
Halibut concentrate	950	620	order 500	583	370	
Do. regenerated	—	no band	142	590	140	425, 402, 378, 357, (340), —, (309), —
Mammalian liver concentrate	56·5	610	37·4	583	23	
Do. regenerated	4·0	610	6·0	583	6·0	420, 399, 376, 357, 341, 326, 309, —
Cod-liver oil C regenerated in $CHCl_3$	blue colour not studied				—	—, 398, 375, 355, 341, 325, (308), —

band (max. 328 $m\mu$) a series of narrow bands with maxima near 425, 399, 376, 357, 340, 324, 308 and 280 $m\mu$. From the data given in Table I it will be seen that after vitamin A and antimony trichloride in chloroform have reacted to form the highest attainable proportion of blue pigment (about 1 minute), a part of the vitamin A can still be recovered unchanged, although an appreciable proportion has undergone decomposition. The narrow absorption bands shown by the new material make the reaction easy to follow with the spectroscope. The procedure described above has been applied to a variety of fish-liver oils, mammalian liver fats and a wide range of concentrates. The wave-lengths of maximum absorption are always the same, indicating that the decomposition is qualitatively independent of the concentration of vitamin A; the intensities of absorption are always of the same order as the intensity of the vitamin A band in the starting material. Although the 328 $m\mu$ band cannot be observed as such in the "regenerated" material, its presence, masked by the highly selective absorption of the new products, can be inferred with certainty from the general shape of the absorption curve in conjunction with the results obtained by applying the colour test to the recovered materials.

It is interesting to note that the maxima for the new products differ noticeably from those recorded previously for the acids obtained by the hydrolysis of liver oils and concentrates [Gillam, Heilbron *et al.*, 1931] particularly after allowing for solvent displacement of wave-lengths.

	λ max. in $m\mu$									
Regenerated products (in chloroform)	425,	399,	376,	357,	340,	324,	308,	280		
Cod-liver oil acids (in alcohol)		392,	375,	350,	330,	316,	302,	281,	269,	259, 235

The similarity of the resolution into narrow bands may indicate a structural resemblance. That the "regenerated" products are not acidic is, however, shown by the fact that no substances showing narrow absorption bands are extracted on washing with either sodium carbonate or sodium hydroxide. Water is also not essential to the appearance of the narrow bands, since they can be recorded in solutions prepared simply by diluting the blue solutions with much chloroform, making no attempt to eliminate the antimony trichloride.

Other methods for obtaining the substances showing narrow bands.

A. When unsaponifiable extracts from liver oils and fats are dissolved in absolute alcohol containing a little dry hydrogen chloride, the ultra-violet absorption spectrum undergoes a considerable change. For example, a 0·005% solution of a rich halibut-liver oil concentrate, dissolved in absolute alcohol, was found to exhibit the normal vitamin A band, the intensity maximum occurring at $E^{1\%}_{1\,cm.}$ 328 $m\mu$, 1100. The large absorption band was completely free from "fine" structure. When, however, the same concentrate in alcoholic hydrogen chloride was examined, it was found not only that $E^{1\%}_{1\,cm.}$ 328 $m\mu$ had fallen to 575, but the selective absorption had been displaced towards the visible

and a succession of narrow bands was recorded, the maxima being at 419, 392, 369, 350 and 333 $m\mu$. The appearance of this set of narrow absorption bands is shown perfectly clearly with even the richest concentrates, and the conclusion cannot be evaded that these bands are characteristic of a material which is either an isomeride of vitamin A or a product very easily derived from vitamin A.

In order to elucidate the mechanism of the action of hydrogen chloride on vitamin A, we have studied the effect of varying the proportions of vitamin A and acid. Using a 0·006 % solution of rich concentrate containing perhaps 50 % vitamin A, when the concentration of hydrogen chloride is low (order $10^{-3} - 10^{-4}\,N$) the narrow bands appear slowly, approaching maximum definition in 1 to 2 days and thereafter gradually disappearing. With a higher concentration of hydrogen chloride ($> N/50$) the narrow bands are fully developed by the time (30 minutes) the spectral absorption can be photographed, but the rate of disappearance appears to be definitely increased. Assuming provisionally that the intensity of the 328 $m\mu$ band for "pure" vitamin A is $E^{1\%}_{1\,cm.} = 1350$, and that the molecular weight is near 300 [Heilbron *et al.*, 1932] it is possible to calculate the relative molecular concentrations of vitamin A and hydrogen chloride. On this basis the range over which our observations extend is [vitamin A]/[HCl] = 1/8000 to 1/0·4. The degree to which the narrow bands can be developed is somewhat variable and there seems to be little doubt that, under the experimental conditions, the substance (or substances) responsible for these bands is somewhat unstable. Many of the differences observed can be readily accounted for by the superposition of the new absorption spectrum on that characteristic of the unchanged vitamin A. The main product, when prepared under favourable conditions, exhibits maxima at 392, 369, 350 and 333 $m\mu$ (see Fig. 2), the 369 $m\mu$ maximum being the most intense. This stage is never attained with very dilute HCl even when in excess over the vitamin, whilst unduly strong acid merely increases the instability of the new product.

The determining factor appears to be the absolute concentration of the HCl rather than the [vitamin A]/[HCl] ratio, since, using the same strength of acid, no difference can be detected in the absorption spectrum whether this ratio is 1/3 or 1/300. On standing, the definition slowly decreases, but this tendency can be arrested, if not entirely avoided, by the addition of sufficient sodium ethoxide solution to neutralise the hydrogen chloride. Similar arrests can be obtained earlier or later in the reaction between vitamin A and hydrogen chloride. It should be remarked, however, that there is no evidence of reversibility, the sodium ethoxide merely tending to stabilise the condition of affairs at the moment it is added.

The best method for obtaining solvent-free material rich in the new absorbing compound is to dissolve 0·5–1·0 g. of a rich concentrate in 100 cc. alcohol and to add enough alcoholic hydrogen chloride to make the solution approximately $N/30$. The mixture is left to stand in the cold for 20 minutes.

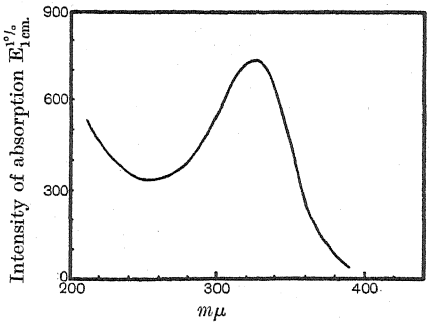


Fig. 2 *a*.

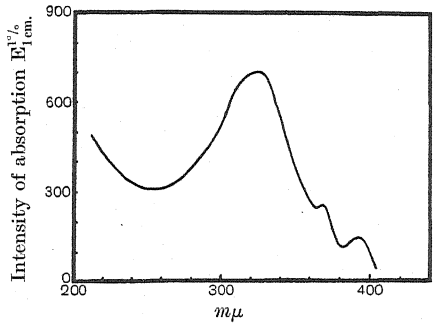


Fig. 2 *b*.

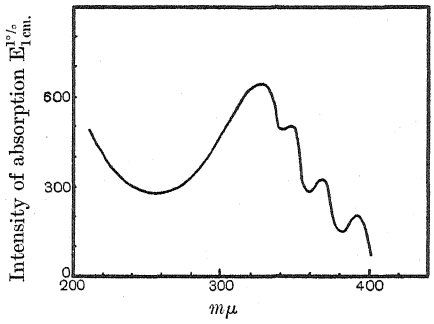


Fig. 2 *c*.

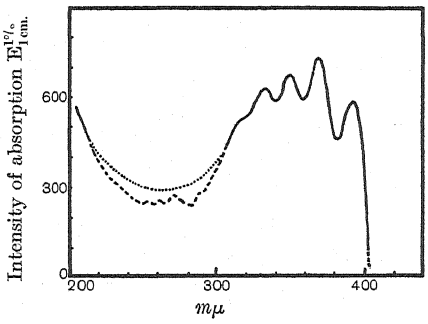


Fig. 2 *d*.

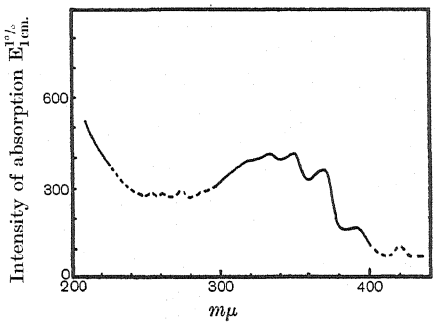


Fig. 2 *e*.

Absorption curves illustrating successive stages in the action of alcoholic hydrogen chloride on vitamin A.

2 *a*. Vitamin A concentrate in abs. alcohol. (Vitamin content about 55 % of that of the richest preparation so far obtained.)

2 *b*. Vitamin A concentrate in alcoholic hydrogen chloride. This curve shows the earliest detectable change.

2 *c*. A later stage in which the alcoholic hydrogen chloride has caused the narrow bands to appear more clearly.

2 *d*. The absorption curve observed at the point at which the narrow bands reach their maximum definition. The curve drawn with a continuous line can be reproduced without difficulty and it should be noticed that the highest absorption occurs at 369 *mμ*. The broken lines indicate that strictly reproducible results in this region of the spectrum are not always obtained.

2 *e*. The absorption curve obtained with vitamin A and alcoholic hydrogen chloride after the mixture (*cf*. 2 *d*) had been left to stand for some days. The poorer definition is worthy of note.

It is then poured into water (400 cc.) and the yellow emulsion extracted several times with small quantities of low-boiling petroleum. After washing with water, and drying over sodium sulphate, the petroleum extract exhibits well developed narrow bands at 392, 369 (highest), 350, and 333 $m\mu$, and ill-defined bands in the regions 316–320, 286–290, 272, 261 and 254 $m\mu$ (these ill-defined maxima are recorded because they have been repeatedly observed, but at the present stage of the enquiry they are much less important than the group 393, 369, 350, 333 $m\mu$). The viscous red-brown oil remaining after removal of solvent exhibits the usual four main bands with great clarity. In an attempt to gain some insight into the mechanism of the reaction leading to this new material, molecular weight determinations, micro-analyses and colour test determinations were carried out and are summarised in Table II.

Table II.

	Vitamin A concentrate	New product
Colour test	$E^{1\%}_{1\,cm.}$ 617 $m\mu$ 3900 580 $m\mu$ 2300	$E^{1\%}_{1\,cm.}$ 617 $m\mu$ 2350 580 $m\mu$ 1400
Micro-analysis	C, 82·8 % H, 10·9 %	C, 82·9, 82·7 % H, 10·4, 10·6 %
Molecular weight (cryoscopic method: solvent benzene)	327	403

The results show that the new product is heterogeneous and that [Heilbron *et al.*, 1932] some polymerisation has probably occurred. The blue value is somewhat higher than would be expected from the ultra-violet absorption.

B. The appearance of narrow bands during the slow high vacuum distillation of rich concentrates is discussed elsewhere. In order to discover how far this effect was due to the prolonged heating, a very rich vitamin A preparation (0·3 g.) was heated in an atmosphere of nitrogen, the temperature being maintained within the limits 120–125°. Small weighed portions were removed from time to time and studied spectroscopically. The results may be summarised as follows. The starting material exhibited the vitamin A band at $E^{1\%}_{1\,cm.}$ 328 $m\mu$, 1100, the absorption curve showing great persistence, as will be realised from the fact that the minimum at 245–250 $m\mu$ occurred at $E^{1\%}_{1\,cm.}$ 300. After 6 hours, the 328 $m\mu$ band had largely disappeared and had been replaced by new absorption in the region 290 $m\mu$. At this wave-length the intensity remained practically constant at $E^{1\%}_{1\,cm.} = 370$ for a further 54 hours' heating, when the experiment was stopped; the definite maximum at 290 $m\mu$ attaining its highest persistence (never very great) after about 10 hours. The minimum was also fairly constant at $E^{1\%}_{1\,cm.}$ 265 $m\mu$, 330. The narrow absorption bands first became detectable after about 10 hours, and reached the highest definition a few hours later, but at best occurred with far less clarity than in the experiments with alcoholic hydrogen chloride or in the fractional slow high vacuum distillation, being little more than fairly sharp inflexions. Substantially, then, the prolonged heating at 120–125° brings about a type of

decomposition associated much more with a substance exhibiting the 290 $m\mu$ maximum than with the narrow bands. If, therefore, the occurrence of these bands in certain distillates is due to heat alone, temperatures above 125° are seemingly essential, and possibly the reaction occurs in the vapour phase. It is interesting to note that while the distillates consisted of pale oils, in our experiments the material grew more and more viscous as the heating progressed, eventually becoming a clear red resin softening at 40–50°. Determinations of the colour test intensities (antimony trichloride) indicated a steady decrease in vitamin A content, but even after 60 hours a perceptible blue colour was given although the intensity had fallen to about 1/40 of the initial value.

Oxidation of vitamin A.

There is general agreement in the literature of the subject that oxygen should be excluded as completely as possible in order to minimise loss during the processes of saponification and extraction of the vitamin from liver oils. During recent months we have repeatedly observed, however, that rich concentrates exhibit a somewhat unexpected resistance to oxidation under certain conditions, and Dann [1931; 1932] has already shown that oxidation of vitamin A concentrates by aeration at the boiling-point in alcohol or alcoholic potash is not achieved in 24 hours. Our own experiments have been carried out on rich halibut-liver oil concentrates dissolved in alcohol. A moderately brisk stream of oxygen from a cylinder was passed through the solution heated under reflux and samples were withdrawn periodically, the vitamin A potency being determined in terms of the intensity of the ultra-violet absorption maximum at 328 $m\mu$. For 8–10 hours the absorption spectrum remained quite constant within experimental error (here $\pm$ 5 %), but after this period the intensity of absorption at 328 $m\mu$ slowly decreased and fine absorption bands with maxima near 392, 369 and 350 $m\mu$ began to appear, not, however, with any great intensity. During the experiment there is a very decided increase in the intensity of absorption on the ultra-violet side of 328 $m\mu$ and this is reflected in a large decrease in persistence. The simplest interpretation of the results is that after a prolonged period of induction slow oxidation occurs coinciding with two changes in spectral absorption, one the appearance of rather unselective absorption in the middle ultra-violet and the other the appearance of highly selective absorption on the long wave side of 328 $m\mu$. The experiments confirm Dann's observation of the high resistance to oxidation of alcoholic solutions of vitamin A, and indicate that the first oxidation product probably exhibits a broad unresolved band near 290 $m\mu$.

As would be expected, ozone is much more effective than oxygen and the vitamin is readily destroyed. In our experiments the object was to ascertain whether any new absorption bands could be recorded after the vitamin had been exposed for a few seconds to small quantities of ozonised oxygen. The reaction proved very difficult to control but certain qualitative observations are of interest. In the first place, exposure of dilute alcoholic solutions of

vitamin A to ozonised oxygen invariably results in a substantial decrease in the intensity of absorption at 328 $m\mu$, but the intensity in the region 250 to 300 $m\mu$ does not fall nearly so rapidly, so that the reaction products must absorb more strongly in the middle ultra-violet than in the region 300–400 $m\mu$. In a number of cases, the exposure to ozonised oxygen was of very short duration, and it seems that the first spectroscopically detectable oxidation (ozonisation) product is characterised by a broad absorption band of small persistence, free from "fine" structure, and exhibiting a maximum at 290 $m\mu$ approx. The intensity of this absorption band is of the same order as that of vitamin A at 328 $m\mu$. In other experiments a maximum at 272 $m\mu$ made its appearance. In all experiments in which a solution of rich concentrate (0·008 %, $E^{1\%}_{1cm.}$ 328 $m\mu$, 1100) was exposed to a fairly slow stream of ozonised oxygen for more than 1 minute at room temperature the resulting solution showed no appreciable absorption over the range 200–500 $m\mu$. In no circumstances was it possible to observe narrow, sharply defined absorption bands as a result of the action of ozone on vitamin A concentrates.

A complete interpretation of the foregoing results is impossible at this stage, but it may perhaps be useful to point out a distinctly interesting analogy emerging from this work and that of Kuhn [1931] on unsaturated compounds [*cf.* also Euler *et al.*, 1932]. Crotonic acid has a broad band with a maximum at 204 $m\mu$, sorbic acid has a similar band with its maximum displaced to 250 $m\mu$, whilst the octatriene and decatetraene acids exhibit bands at 294 $m\mu$ and 329 $m\mu$ respectively (private communication from Prof. Kuhn). Similarly Euler *et al.* [1932] record continuous end absorption on the ultra-violet side of 250 $m\mu$ for sorbic alcohol ($CH_3(CH=CH)_2CH_2OH$), a well defined maximum near 270 $m\mu$ ($\log \epsilon$ 4·75) for octatrienol ($CH_3(CH=CH)_3CH_2OH$) and a maximum near 310 $m\mu$ ($\log \epsilon$ 4·7) for decatetraenol ($CH_3(CH=CH)_4CH_2OH$). Now, it seems significant that the first oxidation product of vitamin A exhibits a maximum near 290 $m\mu$ and the second product a maximum near 272 $m\mu$. By analogy with the data quoted above it is likely that the selective absorption of vitamin A is due largely to a straight chain containing four conjugated double bonds and that the displacement from 310 (decatetraenol) to 328 $m\mu$

Table III.

Material	λ max. ($m\mu$)	*log ε max.
Crotonic acid	204	4·08
Sorbic acid	250	4·40
Octatriene acid	294	4·56
Decatetraene acid	329	4·69
Sorbic alcohol	< 240	order 5
Octatrienol	270	4·75
Decatetraenol	310	4·7
Vitamin A	328	4·5 *circa* (calculated on a molecular weight near 300)
1st oxidation product	290	order 4·5
2nd oxidation product	272	—

* ε is molecular extinction coefficient.

(vitamin A) is due to the joint action of the fifth double bond and the "loading" effect of the ionone ring. Probably, the first action of ozone is to attack one of the double bonds in the side chain, and then selectively to attack a second. It is interesting that only two intermediate stages in the oxidation can be detected spectroscopically. This is not surprising since sorbic acid has its maximum well in the ultra-violet and sorbic alcohol probably in the more extreme ultra-violet.

Table III illustrates the main points referred to above.

Discussion and summary.

The recovery of vitamin A from the blue solution obtained with antimony trichloride by pouring the mixture into much water has been attempted. Partial decomposition of the vitamin occurs, accompanied always by the appearance of narrow absorption bands with maxima at 399, 376, 357 and 340 $m\mu$ (chloroform), and sometimes by additional, less definite maxima, *e.g.* at 425, 324, 308 and 280 $m\mu$. The wave-lengths at the maxima are constant, irrespective of whether either rich or poor oils, or concentrates are used, but the intensities vary roughly with the potency of the material. Recovery of vitamin A, although never quantitative, supports the view that the initial reaction is the formation of a vitamin A-antimony trichloride loose addition product, but secondary (condensation?) processes cannot be excluded, and probably account for the narrow bands. This view is supported by the observation that the similar maxima 392, 369, 350, 333 $m\mu$ (alcohol) can be obtained with greatly enhanced definition by the action of alcoholic hydrogen chloride on rich concentrates, the intensity, persistence, rate of development and permanence depending on the concentration of hydrogen chloride. When the narrow bands are best shown, the 369 $m\mu$ band is the most intense but even under optimum conditions (15°, $N/30$ HCl, 20 minutes) the reaction is incomplete and the product unstable in acid. Neutralisation of the acid with sodium ethoxide arrests the decomposition of the material, but does not effect reversal to vitamin A.

Similar narrow bands, together with a broad continuous band with its maximum at 290 $m\mu$ are shown in certain fractions obtained in slow high vacuum distillation of rich concentrates heated to about 180°. By prolonged heating at 120–125° in an atmosphere of nitrogen the development of narrow bands could not be induced to more than a minute extent, but the vitamin nevertheless decomposed fairly readily, the main product exhibiting a maximum at 290 $m\mu$. At the boiling-point in alcohol, vitamin A is unexpectedly stable to prolonged exposure to a stream of oxygen. After some hours, the 328 $m\mu$ maximum decreases in intensity, the absorption in the region 260–300 $m\mu$ slowly increases and the narrow absorption bands in the near ultra-violet appear, but with comparatively low persistence. Ozonised oxygen rapidly destroys vitamin A with disappearance of selective absorption but compounds with maxima at 290 $m\mu$ and 272 $m\mu$ can be detected in the very early stages.

The data are consistent with the vitamin A molecule containing a side chain with at least four double bonds, which can be attacked in turn by ozone to give products with broad bands.

The hypothesis of ring closure to give hydronaphthalene derivatives as an explanation of the narrow bands will be discussed in a later communication.

We desire to express our thanks to the Department of Scientific and Industrial Research for a grant enabling Mr R. H. Creed to render valuable assistance in the laboratory work..

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CXXXVIII. THE ISOMERISATION OF CAROTENE BY MEANS OF ANTIMONY TRICHLORIDE.

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The stable blue solution (λ max. 583–590 $m\mu$) obtained by the action of antimony trichloride on carotene in chloroform has been poured into water and the organic matter recovered free from antimony. The product is different from, but isomeric with, carotene (compare "regeneration" experiments on vitamin A [Edisbury *et al.*, 1932].

The carotene used (M.P. 180°) was a commercial sample recrystallised from pyridine. Purified in this way carotene exhibits maxima in the spectral absorption curve (solvent, chloroform) at 495, 462, 436, 348 and 280 $m\mu$. The absorption attains its highest intensity at 462 $m\mu$, at which point the molecular extinction coefficient is 102,000 ($E^{1\%}_{1\,\mathrm{cm.}}$ 1900), in agreement with the results obtained previously for the purest carotene [*cf.* Dulière, Morton and Drummond, 1929]. The intensity of absorption at the maximum (585 $m\mu$) in the colour test with antimony trichloride can be expressed as $E^{1\%}_{1\,\mathrm{cm.}}$ 585 $m\mu = 420$.

The method adopted in the "regeneration" experiments was as follows. Carotene (1 g.) was dissolved in chloroform (100 cc.) and treated with a saturated solution of antimony trichloride in chloroform (140 cc.). The mixture was allowed to stand for two minutes and was then poured into water, whereupon antimony oxychloride was precipitated and the original colour of the carotene returned. The oxychloride was dissolved in the minimum quantity of concentrated hydrochloric acid and the mixture left to stand, when the chloroform layer gradually separated. The aqueous portion was shaken with chloroform to recover every trace of extractable material, and the united chloroform extracts were repeatedly washed with water till free from all traces of antimony, and finally dried over sodium sulphate. After removal of solvent by evaporation in a current of nitrogen, a red crystalline residue similar to carotene in appearance was obtained.

This material was then boiled with pentane (200 cc.) and the insoluble residue (fraction A) removed by filtration. The filtrate was concentrated to half-bulk when a further crop of crystals, M.P. 170°, was deposited (fraction B), while complete evaporation of the solvent gave a crystalline residue, M.P. 140° (unsharp) (fraction C). Recrystallisation of A from pyridine raised the M.P. to 185° which was not altered by further purification.

All three fractions of the regenerated product when dissolved in chloroform gave solutions slightly redder in colour than those obtained with carotene at the same dilution. The absorption spectrum in the visible region was characterised in each case by a group of maxima similar to that shown by carotene, but displaced in the direction of the longer wave-lengths. The absorption in the ultra-violet was totally different from that of carotene (see Fig. 1).

Material	M.P.	Wave-length maxima in $m\mu$						
Gross "regenerated"	—	509	482	454	425	389	367	317
Fraction A	185°	512	483	453	(426)	389	—	316
Fraction B	170°	511	480	450	423	389	367	316·5
Fraction C	140°	510	480	450	(423)	—	—	317
Carotene	180°	497	463	436	—	—	350	280

It will thus be seen that the new material is unmistakeably different from carotene in its optical properties. It exhibits a larger number of narrow absorption bands, and in the visible, where the absorption curve is qualitatively similar to that of carotene, the wave-length displacement is very definite. All three fractions exhibit the same maxima and cannot be differentiated qualitatively.

The regenerated product gives a blue solution with the antimony trichloride reagent exhibiting a maximum at 583–587 $m\mu$, practically identical with that of ordinary carotene. The intensity, however, with the sample of highest M.P. did not reach that obtained with the purest carotene recrystallised from pyridine.

Material	Colour test $E^{1\%}_{1\,cm.}$ 585 $m\mu$	Visible absorption $E^{1\%}_{1\,cm.}$ 462 (480) $m\mu$	Ultra-violet absorption $E^{1\%}_{1\,cm.}$ 317 $m\mu$
Carotene	420	1900	—
Regenerated material:			
Fraction A	370	1800	430
Fraction B	365	1800	435
Fraction C	340	1370	330

Without more elaborate purification on a larger scale it cannot be concluded that the intensities of the absorption bands shown by the regenerated product are much different from those shown by carotene itself.

The three main fractions of the regenerated pigment were examined for growth-promoting activity by tests on young rats, the growth of which had been inhibited by feeding on a diet devoid of vitamin A. The basal diet was compounded of

Purified caseinogen ...	20
Rice starch	80
Salt mixture	5
Dried yeast	5

Vitamin D was supplied by the administration of 0·001 mg. irradiated ergosterol twice a week. This, as well as the recovered pigment, was given in the form of a solution in ethyl laurate. The solutions were prepared twice a week and stored in the dark at low temperature. Only one preparation showed any sign of growth-promoting power, namely, that with the highest melting-point (185°). When administered in amounts which in the case of carotene bring about a good resumption of growth, there was an indifferent response (see Fig. 2). The results of tests with larger doses suggested that the material was mainly composed of an inactive substance but that a small amount of an active component, possibly unchanged carotene, was also present.

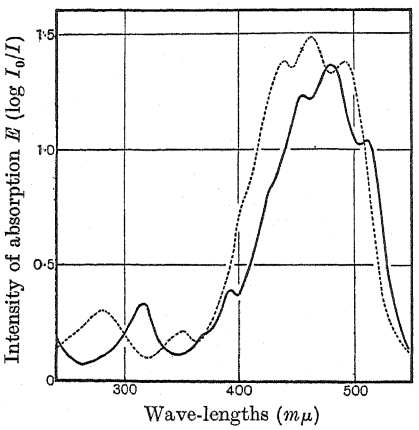


Fig. 1. Spectral absorption curves:
---- Carotene (recryst. pyridine).
Calculated to 0·00078 % (1 cm.) $CHCl_3$.
—— Regenerated product.
Calculated to 0·00075 % (1 cm.) $CHCl_3$.

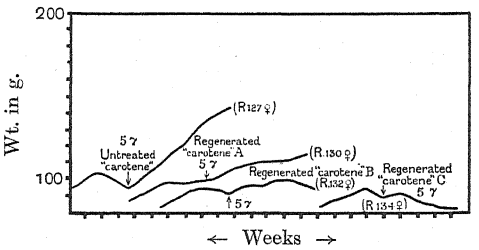


Fig. 2. Biological tests on carotene before and after treatment with antimony trichloride.

The preparations with lower melting-points were devoid of growth-promoting power.

Comparison of the regenerated product with the different varieties of "carotene" studied by Kuhn and Brockmann [1931], and Kuhn and Lederer [1931; 1932] reveals that it is probably identical with *iso*carotene, prepared by the action of either metallic mercury or sodium thiosulphate upon carotene tetraiodide.

	M.P.	Absorption maxima in CS_2, $m\mu$				Growth-promoting activity
α-Carotene	174–175°	511	478	448	—	good
β-Carotene	180–181°	520	485	454	—	good
*iso*Carotene	185–186°	543	503	472	—	nil
Regenerated product	185°	540	505	478	445	nil

SUMMARY.

1. When the blue solution obtained by mixing antimony trichloride and carotene in chloroform is poured into water, the original colour of the carotene reappears in the chloroform layer from which red crystals can be isolated.

2. The absorption spectrum of the recovered product has been determined in chloroform and carbon disulphide. Both in the visible and ultra-violet regions it is quite different from that of carotene.

3. The properties of the substance agree with those of *iso*carotene.

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CXXXIX. CHARACTERISTICS OF HIGHLY ACTIVE VITAMIN A PREPARATIONS.

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In a series of papers emanating from these laboratories during the past five years unequivocal spectroscopic data for vitamin A have been established, and the ground has been cleared for a final comprehensive attack on the problem of isolating the vitamin and establishing its constitution. The present communication embodies the results of work carried out during the last 18 months, and leads us to the view that a definite solution of the problem is now in sight, a view supported by the recent highly important and illuminating publications of Karrer, Morf and Schöpp [1931, 1, 2], to which further reference will be made later.

Vitamin A, both in liver oils and concentrates, exhibits a broad continuous absorption band in the ultra-violet, extending from 260 to 380 $m\mu$, and exhibiting a maximum near 328 $m\mu$. The intensity of this band can easily be measured and used as a definite quantitative test for the potencies of oils and concentrates. Vitamin A also shares with a variety of polyene substances the power of giving a blue solution with a saturated solution of anhydrous antimony trichloride in chloroform. This colour test gains in specificity when the spectral absorption of the blue solution can be accurately described. As a matter of convenience attention may be concentrated on the points of maximum absorption described in terms of intensity and spectral location[1]. Thus the blue solution obtained with vitamin A exhibits two maxima at 617 and 583 $m\mu$ respectively, the former being generally much more intense than the latter, which it tends to mask. In unsaponified oils the maxima are displaced to 606 and 573 $m\mu$ respectively, and here the longer wave-length absorption

[1] The notation used in this paper is necessarily a little different from that employed in spectroscopic studies on pure substances. The usual method is to state the maximum molecular extinction coefficient ϵ, defined by $E = \log I_0/I = \epsilon cd$. ($I_0$ is intensity of incident light, I is intensity of emergent light, E is extinction, c = molar concentration, d = thickness in cm.) In this work $E^{1\%}_{1\text{ cm.}}$ refers to $\log I_0/I$ at a wave-length maximum, using a solution containing 1 % of vitamin-containing substance and a 1 cm. cell.

suffers considerable but variable diminution in intensity as a result of the inhibiting action of certain normal constituents of fish-liver oils.

In a number of oils and concentrates the presence of additional chromogenic substances manifests itself in the colour test by giving rise to other maxima, among which one at 693 $m\mu$ occurs with considerable regularity [Morton, Heilbron and Thompson, 1931]. With such criteria of vitamin A available it seemed obvious that, provided a satisfactory method of isolation of the vitamin could be achieved, pure preparations from the livers of various species of both fishes and of mammals should all exhibit the maxima at 328, 617 and 583 $m\mu$, each at its own limiting maximum intensity, if all three were indeed properties of vitamin A itself. On the other hand, if any one of the three maxima appeared with relatively diminished intensity, the interpretation would have to be reconsidered. It by no means necessarily follows, however, even if constant values are reached, that vitamin A has been obtained as a homogeneous substance, although one condition of purity is apparently satisfied. It is this stage which we have now reached in our investigations; concentrates of equal potency derived from different sources have been obtained and have proved incapable of being increased in potency by any process calculated to result in a separation of the constituents of a mixture.

Table I.

Material	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	$E^{1\%}_{1\,cm.}$ 617 $m\mu$	$E^{1\%}_{1\,cm.}$ 580 $m\mu$
Average cod-liver oil	0·78*	0·92	0·70
Typical halibut-liver oils (*a*)	4·25	7·5	6·0
,, ,, (*b*) winter catches	3·5	6·2	4·8
,, ,, (*c*)	15·6	32·0	16·7
Total non-sap. (9 %) from (*c*)	380	720	460
Rich halibut oil (*d*)	35	70	47
Non-sap. of (*d*) (approx. 2 %) after freezing at −50°	1150	3300	1800
Rich halibut oil (*e*)	70	148	85
Total non-sap. (8 %) of (*e*)	550	1150	600
(*e*) deprived of sterol at −10° (4·7 %)	1060	2210	1110
(*e*) after treatment at −50°	1150	3020	1450

* Equivalent to 9 Carr-Price units on oil and 17·2 on non-sap.

Earlier work had convinced us that if vitamin A were to be prepared in a state approaching purity, it was certainly desirable, if not indeed absolutely essential, to start from highly active oils. In conjunction with Dr J. A. Lovern of the Torry Research Station, Aberdeen, a large number of fish-liver oils of different species have been examined under conditions most favourable to conservation of the vitamin and, as a result, enormous variations in potency have emerged not only between the oils from various species but also between different specimens of oil from a single species. Apparently the potency of some fish-liver oils is much lower in the winter than in summer, but this seasonal variation provides, we believe, only a partial explanation of the wide deviations. From the mass of data in our hands, one fact very soon impressed

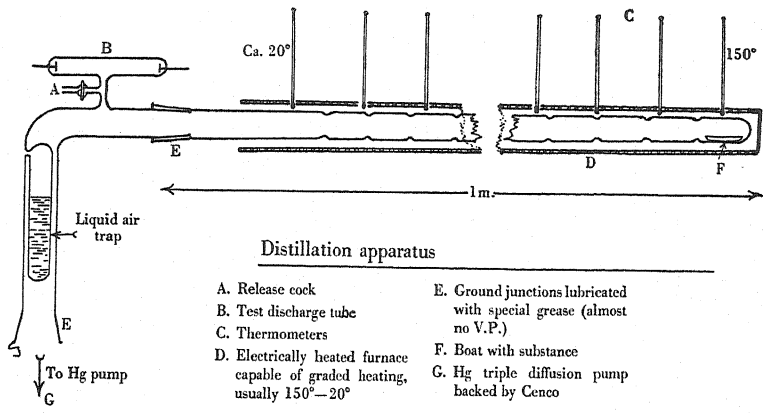
itself, namely that of all fish-liver oils studied, that from the halibut is outstanding in showing occasionally unusually high potency, and it is such oils that we have generally employed as starting material in this investigation. The importance of a careful selection of raw material will be evident from Table I, which illustrates by means of the spectroscopic maxima the wide variations observed in halibut-liver oils.

The general method of saponification adopted by us consisted in heating the oil (500 g.) with 12 % alcoholic potash (1250 cc.) at 75° for 1 hour in an atmosphere of nitrogen under slightly reduced pressure, during which period approximately half the alcohol distilled over. The cooled solution was diluted with distilled water (6000 cc.) and the unsaponifiable matter removed by repeated extraction with ether. After drying over anhydrous sodium sulphate and removal of solvent, the residue was dissolved in hot methyl alcohol (150 cc.) and left to stand, when the bulk of the sterols (mainly cholesterol) present in the mixture separated and were removed at the pump. The filtrate was reduced to half bulk in an atmosphere of nitrogen, and cooled to −10°, when more sterol crystallised and was removed. The residual solution was then cooled to about −50° in a mixture of solid carbon dioxide and ether, and after standing for some time rapidly filtered through a jacketed sintered filter maintained at the same temperature. The residue, which had a comparatively low vitamin content, was rejected. The filtrate was diluted largely with water and extracted with light petroleum (B.P. 40–50°) and the solvent finally removed from the dried extract, which was then heated at 70° in a high vacuum. The stages in the concentration of the vitamin, starting from two rich halibut oils (*d*) and (*e*) are reproduced in Table I.

An estimation of the sterol content of (*e*), after treatment at −50°, by means of digitonin showed that approximately 3 % was present. Resaponifications of different samples with both sodium ethoxide and sodium methoxide were carried out, but these failed to produce products of increased potency. For the further concentration of the vitamin attempts were made to separate it both by methods involving the distribution between two solvents and also by adsorption on kieselguhr, calcium carbonate and kaolin, but these processes failed to effect any definite increase in potency as measured spectroscopically. It was therefore decided to resort to distillation in a very high vacuum. Attempts to distil vitamin A have previously been reported by Drummond and Baker [1929], who employed pressures near 0·01 mm., but found that under these conditions the vitamin was largely destroyed in the process. In the hope of avoiding this decomposition a special apparatus was constructed (see Fig. 1) whereby fractional distillation of the already very rich concentrates could be carried out at a pressure certainly below 0·0001 mm. The halibut concentrate (10 g.) was introduced into the boat, which was then heated to between 150 and 155°, when distillation commenced. The main fraction, which formed a heavy golden-yellow oil (*c* in Table II) was collected over a period of 3½ hours. A spectrographic examination of this apparently

Table II.

Material	Condensation temp.	Weights of fractions g.	$E^{1\%}_{1\,cm.}$ 617 mμ	580 mμ	328 mμ	
Sterol-freed non-sap. from halibut-liver oil	—	10	2350	1550	1000	
Distillation fractions:						
(*a*)	25–50°	0·14	480 (624 mμ)	400 (567 mμ)		The ultra-violet absorption shows a clear maximum at 285, a pronounced inflexion at 330 and a small inflexion near 371 mμ $E^{1\%}_{1\,cm.}$ {285 mμ = 271 / 330 mμ = 203
(*b*)	50–80°	0·11	410	340		$E^{1\%}_{1\,cm.}$ {290 mμ = 250 maximum / 328 mμ = 170 inflexion. There are feeble indications of finer structure in the selective absorption.
(*c*)	80–110°	4·9	1760	1160		The general shape of the absorption curve is similar to the normal vitamin A curve, but more selective absorption is superimposed, sharp maxima occurring at 393, 368, 348, 330, 313·5 and 297 mμ $E^{1\%}_{1\,cm.}$ 330 mμ = 710
(*d*)	110–120°	0·57	600	400		The same fine structure is shown $E^{1\%}_{1\,cm.}$ 330 mμ = 265
(*e*) (semi-solid yellow wax; crystals of cholesterol)	120–140°	0·15	250	160		In addition to an inflexion near 370 mμ, the absorption appears to consist of a fusion of two continuous bands with maxima respectively at 330 and 290 mμ. *E* in each case approx. 100. Small quantities of substances exhibiting the narrow bands also occur.
(*f*)	140–155°	4·2	180 (610 mμ)	180 (578 mμ)		A very flat maximum occurs at 290 mμ. $E^{1\%}_{1\,cm.}$ = 65, but there is little evidence of the narrow bands



Distillation apparatus

A. Release cock
B. Test discharge tube
C. Thermometers
D. Electrically heated furnace capable of graded heating, usually 150°–20°
E. Ground junctions lubricated with special grease (almost no V.P.)
F. Boat with substance
G. Hg triple diffusion pump backed by Cenco

Fig. 1.

Table III.

Material	B.P.	Time of collection (min.)	$E^{1\%}_{1\,cm.}$ 328 $m\mu$	617 $m\mu$	580 $m\mu$	C.P. blue value	Analysis % C	% H
A. Halibut non-sap. before distillation (12·3 cc.)	—	—	1000	2350	1550	50,000	—	—
Fractions:								
(*a*) 1·5 cc.	120–137°	15	930	2400	1270	53,000	—	—
(*b*) 2·0 cc.	137–138°	12	1190	3000	1600	54,000	—	—
(*c*) 3·7 cc.	138–139°	85	1200	3160	1470	55,000	82·7	11·1
(*d*) 0·75 cc.	139–180°	13	810	2470	1160	50,000	—	—
(*e*) 2·25 cc.	200–225°	30	750	1810	1020	47,000	81·6 81·2	11·5 11·2
(*f*) Solid	225–310°	38	*	—	—	5,500	80·7 80·6	10·4 10·0
B. Concentrate: before distillation (24 cc.)	—	—	1150	3020	1450	57,000	84·2 84·1	11·3 10·9
after distillation, main fraction (12 cc.)	137–138·5°	57	1300	3680	1860	64,000	82·5 82·5	11·1 10·7
C. Another concentrate: before distillation (6·4 g.)	—	—	1100	3250	1950	50,000	—	—
after distillation:								
(*a*) 0·7 g.	130–137°	10	—	—	—	52,000	—	—
(*b*) 2·5 g.	137–138°	15	1300	3710	1850	60,000	82·4	11·0
(*c*) 1·0 g.	138·5–170°	15	—	—	—	54,000	—	—

* Max. stronger than 617 $m\mu$ max.

undecomposed distillate revealed that actually readily detectable changes had occurred, for in place of the typical continuous ultra-violet absorption band at 328$m\mu$ an absorption curve showing well defined narrow bands was now exhibited. These bands were also found when the vitamin was dissolved in alcoholic hydrogen chloride and in the material obtained on the regeneration of a vitamin A concentrate from the blue antimony trichloride colour test [Edisbury *et al.*, 1932]. The experiment was repeated with a richer starting material ($E^{1\%}_{1\,cm.}$ 328$m\mu$ = 1150) with similar results; it thus appeared plausible that the molecular rearrangement involved might be due to the prolonged period of heating, possibly combined with some catalytic effect due to the large surface of glass with which the vapour comes into contact. With the very helpful collaboration of Dr F. H. Carr and Mr W. Jewell of British Drug Houses, Ltd., the distillation of rich halibut oil concentrates was also carried out in a new type of molecular still, whereby rapid fractional distillation could be effected at a pressure below 0·00001 mm. Under these conditions the vitamin distils without decomposition or molecular change, giving a main fraction, B.P. 137–138°, having considerably enhanced potency. The results of typical distillations are summarised in Table III. Redistillation of the best fraction resulted in a slight decrease of potency as measured

spectroscopically and it would thus seem that the method had been utilised to the point of maximum efficacy. In order to test this point on different material a sturgeon-liver oil was employed which, although initially not particularly rich in vitamin A, after saponification and freezing provided an unsaponifiable fraction of very high potency. The distillation of this is recorded in Table IV, from which it will be seen that the spectrographic values are in excellent agreement with those obtained from the halibut concentrates.

Table IV.

Material	B.P.	Time of collection (min.)	$E^{1\%}_{1\,cm.}$ 328 mμ	617 mμ	580 mμ	C.P. blue value	Analysis % C	% H
A. Sturgeon-liver oil	—	—	18	39	19	—	—	—
Total non-sap. (4·8 %)	—	—	200	500	290	—	—	—
Non-sap. after treatment at −50° (1 %) (6·2 g.)	—	—	1080	3380	1680	57,000	82·1 82·3	10·7 10·5
After high vac. distillation:								
(1) 1 cc.	120°	15	—	—	—	—	—	—
(2) 1 cc.	120–137°	5	1300	3950	1990	—	—	—
(3) 2·8 cc.	137–138°	12	1330	4200	2130	64,000	82·8 82·6	10·9 10·7
B. Mammalian-liver fat	—	—	235	780	470	11,000	—	—
Non-sap. after treatment at −50° (20 %) (9·2 g.)	—	—	1000	2750	1370	—	82·4	11·1
After distillation: Fraction:								
(1) 2·7 g.	143–149°	10	1250	3620	2350	50,000	82·7	11·1
(2) 1·2 g.	149–150°	10	—	—	—	—	—	—
(3) 0·9 g.	150–165°	10	—	—	—	—	—	—
(4) 4·2 g.	Above 165°	—	—	—	—	4,200	—	—

In order to extend these results it seemed desirable to attempt a concentration of vitamin A from an oil of mammalian source, and we are indebted to Messrs Lever Brothers Ltd. for kindly placing a partially concentrated mammalian extract at our disposal. This was again worked up in the manner already described and gave a highly viscous dark unsaponifiable fraction, the spectrographic data for which (see Table V) were in good agreement with the values obtained from similar fractions of the halibut and sturgeon concentrates. Owing to the very high viscosity of the product the distillation into fractions in this case was rather less sharp, but it will be observed that the main fraction again gives substantially the same values for the 328, 617 and 580 mμ bands as with the fish-liver distillates. By thus obtaining from totally distinct sources concentrates of constant potency a considerable advance has been made. Certain difficulties still remain before proof of homogeneity in the highly active fractions can be affirmed. In the first place, the evidence

Table V.

Material	$E^{1\%}_{1\,cm.}$ 328 mμ	617 mμ	580 mμ	Analysis % C	% H
Halibut-liver oil non-sap.	1000	2350	1550	—	—
Small scale adsorption exp. (middle layer) (3 g.)	1100	2730	1470	81·3 81·2	10·7 10·5
Large scale adsorption exp. (middle layer) (17·5 g.)	1050	2470	1220	—	—
Halibut-liver oil non-sap. distillate	1300	3710	1850	82·4	11·0
Adsorption of distillate	1350	4350	2350	83·2	10·8
Repeat adsorption	1330	4320	2320	82·9	10·6
Sturgeon-liver oil non-sap.	1000	2500	1250	81·4	10·6
Adsorption (middle fraction)	1100	2850	1470	81·9	10·6
Repeat adsorption	835	2160	1430	81·7	10·9
Sturgeon-liver oil non-sap. distillate	1330	4200	2130	82·8 82·6	10·9 10·7
After adsorption (middle layer)	Fine structure	1950	1600	85·8 85·5	10·6 10·5
Mammalian-liver oil non-sap.	1000	2750	1370	82·4	11·1
After adsorption (middle layer)	1300	3810	1995	83·1	11·0
Repeat adsorption (middle layer)	—	1450	1140	83·1 83·4	10·3 10·4
Mammalian non-sap. distillate	1250	3620	2350	82·7	11·4
Adsorption (middle and bottom fractions)	1320	3920	2500	82·8	10·9

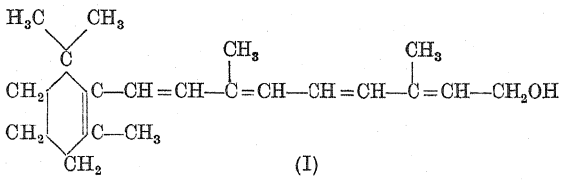
Table VI.

Material		$E^{1\%}_{1\,cm.}$ 328 mμ	617 mμ	580 mμ	693 mμ	Analysis % C	% H
Halibut non-sap. (best distillate)		1300	3680	1860	370	82·5 82·5	11·1 10·7
Another halibut non-sap. distillate (after adsorption)		1350	4350	2350	900	83·2	10·8
Sturgeon non-sap. (best distillate)		1330	4200	2130	1020	82·8 82·6	10·9 10·7
Mammalian non-sap. (after adsorption)		1300	3810	1995	No band	83·1	11·0
Mammalian non-sap. distillate		1250	3620	2350	No band	82·7	11·4
Prof. Karrer's halibut preparations (Liverpool measurements)	(*a*)	1350	4130	2250	364	83·64 83·8	11·1 10·8
	(*b*)	1250	3250	1600	300	—	—

which has been adduced by Heilbron, Gillam and Morton [1931] concerning the complex nature of the reaction of antimony trichloride remains unshaken, and no satisfactory explanation of the highly selective inhibition of the 617 mμ band, a phenomenon which persists in the richest distillates, is yet forthcoming [Morton, 1932]. Even were this difficulty removed a further source of uneasiness arises in the variable intensity of the absorption band with head at 693 mμ in the colour test. The values for this band for different purified concentrates are summarised in Table VI and whereas with sturgeon oil the band was clearly seen at all stages in the concentration at approximately one quarter of the intensity of the 617 mμ band, with halibut-liver oils its intensity varied according to the starting material from one-tenth to one-quarter of that of the 617 mμ band. In the case of the mammalian concen-

trates, which are otherwise indistinguishable from the richest liver oil concentrates, no selective absorption at 693 $m\mu$ could be observed either in the crude extract or in the purified distillates. It is thus difficult to resist the conclusion that even the richest concentrates so far obtained are liable to contain variable quantities of a substance which is chromogenic towards antimony trichloride but is different from vitamin A itself.

While this work was in progress two papers appeared by Karrer, Morf and Schöpp [1931, 1, 2] in which an account is given of the preparation of a vitamin concentrate which, according to Euler [1931] gives a Carr-Price value of 130,000. The method employed by Karrer and his collaborators for the final concentration of the vitamin consisted in adsorption of the sterol-free unsaponifiable fraction of the liver oils of both halibut and *Scombresox saurus* (Saury Pike) on Merck's specially prepared alumina on the lines of Tswett's chromatographic analysis. The purified product, which the authors consider may be regarded as approximately homogeneous, was obtained as a pale yellow viscous oil which gave analytical results in good agreement with either the formula $C_{20}H_{30}O$ (5 double bonds) or $C_{22}H_{32}O$ (6 double bonds). On catalytic hydrogenation the vitamin was found to take up a volume of hydrogen equivalent to 4·9 mols calculated on a molecular weight of 286. Treatment with ozone gave geronic acid, in amount indicating the presence of one ionone ring, whilst quantitative oxidation with permanganate and chromic acid produced acetic acid in amounts corresponding respectively to 9·7 % and 16·3 % C-methyl. From these results the authors favour formula (I) as probably representing the structure of vitamin A.



(I)

The wide discrepancy between the Carr-Price value recorded by Euler [1931] for the purified vitamin of the above authors and the value of about 65,000 units given for our distilled preparations led us to question whether the distillation method was in reality producing as rich a vitamin A concentrate as that obtained by the chromatographic process of fractionation. We therefore decided to test this method for ourselves, using the Merck "Fasertonerde" employed by Karrer and his colleagues. The apparatus used by us consisted of a long glass tube *T* (Fig. 2) filled to a height of 75 cm. with the fibrous alumina previously dried at 150–200° in a current of carefully purified nitrogen. Nitrogen was first repeatedly drawn through the apparatus so as to ensure complete removal of air. A solution of the selected concentrate (2·5 g. in 25 cc. light petroleum) was then introduced at *S* and the pressure reduced slightly so that on opening the cock t_3 it could be drawn into the adsorbing material. The solution, which penetrated the column to a depth of

about 20 cm. was now slowly washed down the whole column by introducing further quantities of light petroleum into *S*. The apparatus was then disconnected and the tube *T* cut so that the adsorbent was divided into three equal parts. Each section was then separately extracted with hot methyl alcohol (50 cc.), the extract diluted with distilled water (200 cc.) and the vitamin re-extracted with light petroleum.

Table V gives a summary of the results obtained in applying this method of purification to sterol-free vitamin A concentrates. It will be seen that

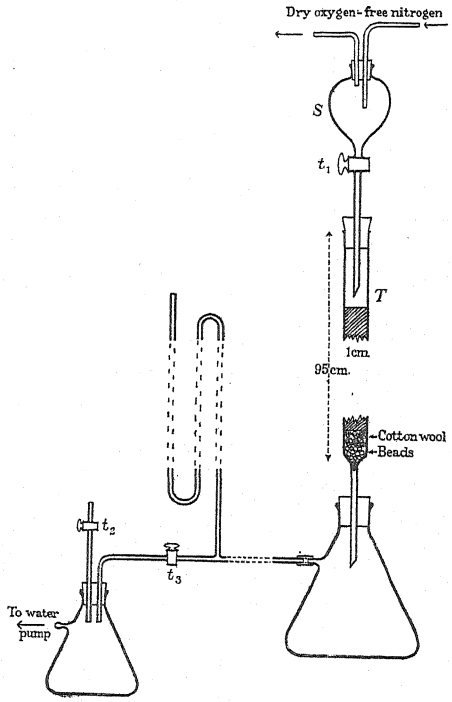


Fig. 2. Apparatus used in the experiments on selective adsorption.

although very definite concentration of the vitamin is effected the results prove that the method possesses no real advantage over that of distillation in a high vacuum. In our experience the method is only applicable to small quantities of material, and further, as measured spectroscopically, the effects of a second fractionation are scarcely perceptible. The increase from 9100 to 10,500 C.L.O. units recorded by Karrer, Morf and Schöpp [1931, 1, 2] as a result of a second adsorption treatment of this concentrate is more probably due to the removal of traces of inhibitors, which, as is well known, greatly modify the blue colour reaction, than to a significant increase in the vitamin A content.

In order to ascertain finally whether the adsorption method was capable

of effecting concentration beyond the highest point attained by distillation we have submitted the main distillates both from the halibut- and the sturgeon-liver oils to the chromatographic process, and have observed either a definite loss of potency or a complete failure to raise the potency beyond the point $E^{1\%}_{1\,\text{cm.}}$ $328m\mu = 1350$. In the case of the richest sturgeon distillate the application of adsorption actually effected an obvious decomposition of the vitamin as evidenced by the appearance of fine structure in place of the smooth $328m\mu$ band (see Table V). This failure to enrich the product was also confirmed on a mammalian concentrate.

As our experiments on the purification of vitamin A by the chromatographic method failed to account for the discrepancies in the Carr-Price values previously referred to, we approached Prof. Karrer, who very kindly supplied us with two specimens of his richest concentrate. A detailed spectrographic examination of these proved that they do not differ appreciably, except as regards the intensity of the $693m\mu$ band discussed elsewhere in this paper, from those prepared by us. The spectroscopic magnitudes for the richest vitamin A concentrates together with Prof. Karrer's two preparations are summarised in Table VI, from which it will be seen that the maximum value ($E^{1\%}_{1\,\text{cm.}}$ $328m\mu$) so far obtained is 1350.

Preparation of concentrates from cod-liver oil.

Emphasis has already been laid on the necessity for using highly potent liver oils as starting materials if concentrates of the highest magnitude of potency are required. That these conditions must be strictly observed is well exemplified in Table VII, where the results of applying the adsorption method of purification to a cod-liver oil initially several times as potent as an average specimen are recorded. After freeing the unsaponifiable matter from sterol by cooling to $-50°$ the potency does not exceed one-third of the maximum value. Fractional adsorption on alumina gives in the first separation the fraction of

Table VII.

No.	Material	$E^{1\%}_{1\,\text{cm.}}$ 328 $m\mu$	617 $m\mu$	580 $m\mu$	693 $m\mu$	Analysis % C	% H
1	Cod-liver oil	4·7	10·4	7·7	—	—	—
2	Total non-sap. (1·4 % approx.)	255	795	425	120	—	—
3	Residue obtained after freezing out sterol at $-10°$	—	940	550	150	—	—
4	Material frozen out at $-50°$	—	350	200	—	—	—
5	Filtrate from (4)	420	1350	800	—	79·6	12·5
6	Fractional adsorption of (5) on fibrous alumina:						
	(*a*) Top portion	—	280	220	—	—	—
	(*b*) Middle portion	400	700	520	105	81·7	10·8
	(*c*) Bottom portion	600	1780	1110	190	—	—
7	Second fractional adsorption using 6 (*c*):						
	(*a*) Middle portion	—	1860	1250	—	79·9	11·0
	(*b*) Bottom portion	—	375	425	—	81·2	10·8

highest potency in the bottom layer of the tube, and even after repetition of the purification process the potency fails to exceed about one-half the maximum value.

Properties of distilled vitamin A.

The distilled product (from all concentrates) forms a pale yellow viscous oil which becomes mobile on warming. It is readily soluble in organic solvents, somewhat more so in methyl than in ethyl alcohol. The vitamin, as obtained after distillation, is unexpectedly resistant to aerial oxidation but is highly susceptible towards acid media [Edisbury *et al.*, 1932]. On standing, however, even in a sealed tube in the dark, a gradual decrease in potency occurs. Thus both with our best materials and that supplied by Prof. Karrer losses up to 10 %, as measured spectroscopically, occurred in less than a month. Analytical data for the highly active distillates and also for the concentrates prepared by the adsorption method where $E^{1\%}_{1\,\mathrm{cm.}}$ 328 $m\mu$ has reached its highest value are given in Table VI, and it will be noted that the results on the whole are in fair agreement with the values required for $C_{20}H_{30}O$. The carbon values of our preparations are somewhat lower than those recorded by Karrer, Morf and Schöpp [1931, 1, 2] or found by us for Prof. Karrer's most active preparation, despite the fact that the absorption data are in outstandingly good agreement. In some cases distillation, while increasing the optically determined potency, seems to effect a slight decrease in the carbon percentage (compare Table III). An unexplained anomalous result was obtained with the sturgeon-oil distillate, which, when an attempt was made to purify it further by adsorption on alumina, gave a resin of low vitamin potency, the carbon value for which, however, was considerably higher than that found for the untreated distillate (Table V). Molecular weight determinations were carried out with the distillate from each species examined according to the Smith and Young modification [1927] of the Rast micro-method and gave as a mean result the value 312, whereas cryoscopic determinations using benzene as solvent gave a mean value of 327. These results are in good agreement with the value of 330 obtained by Karrer by the Rast method, and with the value of 333 calculated by Bruins, Overhoff and Wolff [1931] based on diffusion experiments (mol. wt.: $C_{20}H_{30}O$, 286; $C_{22}H_{34}O$, 314).

Hydrogenation.

Whereas crude vitamin A concentrates are strongly resistant to catalytic hydrogenation, the distilled products can be hydrogenated fairly readily using either palladium or Adams's platinum oxide. The absorption is rapid up to the point when about two-thirds of the total hydrogen is absorbed, after which the rate slows down considerably, and hydrogenation is completed only after many hours and with addition of fresh catalyst. In our experiments we have found that the volume of hydrogen absorbed corresponds to 4·5 molecules, based on $C_{20}H_{30}O$ (5 double bonds) or 4·9 on the less probable $C_{22}H_{32}O$ (6 double bonds) formula. Analysis of the crude hydrogenation pro-

duct gave, in the case of two halibut distillates and the mammalian distillate, the following values respectively: (*a*) C, 79·9; H, 13·3; (*b*) C, 79·8; H, 13·1; (*c*) C, 80·9; H, 12·8 %. In the case of (*b*), fractional distillation in a high vacuum gave a main fraction, B.P. 168–172°/0·06 mm., and a small amount of a higher boiling fraction, B.P. 180–210°/0·08 mm., from which crystalline material separated on long standing. The solid was removed by filtration and after crystallisation from methyl alcohol was obtained as a colourless wax, M.P. 50–53° (Found C, 75·6, 75·8; H, 13·0, 12·9 %). The quantity of this material at our disposal was unfortunately too small to allow of its further investigation but it is our intention to study the hydrogenation of larger quantities of distilled vitamin A so soon as fresh starting material becomes available. The main liquid distillate was tested with tetranitromethane and found still to be slightly unsaturated, as evidenced by the production of a definite yellow colour. It was accordingly rehydrogenated in acetic acid solution with Adams's platinum oxide, when a further quantity of hydrogen was absorbed. The product was again distilled in a high vacuum, and distilled almost wholly at 158–163°/0·04 mm. No colour was given with tetranitromethane, and the product, which was obtained as a thick colourless oil, was again analysed. (Found: C, 81·3; H, 13·0; $C_{20}H_{40}O$ requires C, 81·0; H, 13·6 %.)

Iodine values.

A series of iodine values was determined according to Rosenmund and Kuhnhenn's method [1923] and gave 306, 315, 324 on three different samples. (Iodine value for $C_{20}H_{30}O$ (5 double bonds), 448.) It would seem from these results that the reagent attacks the ethylene linkage of the ionone ring only extremely slowly if at all. Evidence in favour of this assumption may be adduced from experiments on β-ionone when the following results were obtained: (*a*) 5 min. contact with Br reagent 198, 190, (*b*) 20 min. 190, 196, (*c*) 60 min. 231, 234. (Iodine value for $C_{13}H_{21}O$ (2 double bonds), 264.)

Preparation of esters.

Karrer, Morf and Schöpp [1931, 2] have described the preparation of a *p*-nitrobenzoate and an acetate of vitamin A, neither of which is recorded as having been obtained crystalline. We have ourselves prepared a benzoate by the following method. 15 g. of halibut concentrate were dissolved in pure dry pyridine (25 g.) and freshly distilled benzoyl chloride (14 g.) slowly added to the solution. After standing at room temperature for some time, the mixture was heated for 1 hour on a water-bath at 80° in an atmosphere of nitrogen. After cooling, the solution was diluted with water, extracted with ether, and the ethereal extract repeatedly washed successively with dilute sodium carbonate solution and water, and dried over anhydrous sodium sulphate. The deep yellow, thick oil obtained after removal of solvent was repeatedly extracted with hot methyl alcohol until the extracts were no longer coloured. The residual oil (6·2 g.) was taken up in ether, dried, and after removal of

solvent subjected to high vacuum distillation when, as shown in Table VIII, a main fraction was collected distilling between 188 and 198° without noticeable decomposition. The distilled benzoate formed a pale yellow viscous oil which could not be induced to crystallise. (Found: C, 82·2; H, 9·2 %. $C_{20}H_{29}O.COC_6H_5$ requires C, 83·0; H, 8·7 %.) Attempts have also been made to prepare solid esters using dinitrobenzoyl chloride, diphenic anhydride, phthalic anhydride and the acid chloride of anthraquinone-1-carboxylic acid, without success. Further experiments are now being carried out on these lines in the hope of obtaining solid derivatives of the hydrogenated alcohol or its oxidation products.

Table VIII.

Material	Temp. of distillation	Time of distillation, min.	$E^{1\%}_{1\,cm.}$ 328 mμ	617 mμ	580 mμ	Carr-Price blue value	Analysis % C	% H
Halibut-liver oil concentrate used	—	—	1150	3020	1450	57,000	84·2	11·3
Total benzoylated product	—	—	710	1720	860	—	82·1 82·3	8·7 9·0
Benzoylated product (insol. in methyl alcohol) (6·2 g.)	—	—	750	1920	910	40,000	—	—
After high vacuum distillation: Fraction:								
(1) 0·2 g.	165–180°	—	Quantity too small for full examination					
(2) 1·5 cc.	170–190°	20	780	2020	1010	34,000	—	—
(3) 2·5 cc.	195–198°	60	780	2250	1200	37,000	82·2	9·2
(4) 0·3 g.	Over 200°	—	290	550	345	15,000	—	—
(5) 0·7 g. residue	Not volatile at 300°	—	145 (300 at 280 mμ)	105	160	—	—	—

Ozonisation.

We have repeated the ozonisation experiments described by Karrer, Morf and Schöpp [1931, 1, 2] using a halibut-liver oil distillate. Working under the conditions specified by the above authors we have isolated geronic acid in the form of its semicarbazone, which after recrystallisation from ethyl acetate was obtained in colourless needles, M.P. 163–163·5°. The isolation of this acid fully confirms the presence of the ionone ring in the vitamin A structure [see also Heilbron, Morton and Webster, 1932].

Biological potency of the vitamin concentrates.

The physiological potency of certain of the distillates was tested by administration of measured amounts to young rats which had been fed upon a basal diet deficient in vitamin A until growth had ceased. The composition of the ration was

Purified caseinogen	20
Rice starch	80
Salt mixture	5
Dried yeast	5

Vitamin D was supplied by the separate administration of 0·001 mg. of a tested preparation of irradiated ergosterol twice weekly. Both this supplement and the fractions being tested for vitamin A potency were given in the form of solutions in freshly-distilled ethyl laurate. The test solutions of vitamin A concentrates were made up twice weekly and were stored at low temperature in the dark.

The concentrate prepared from halibut-liver oil and fractionated by distillation ($E^{1\%}_{1\,cm.}$ 328$m\mu$ = 1300) promoted growth in doses of 0·1γ daily and cured xerophthalmia in 48 hours (Fig. 3). Smaller doses of this concentrate were not tested but in the case of the distilled concentrate from the sturgeon-liver oil an improvement in general condition and a slow resumption of growth occurred when daily doses as low as 0·025γ were given. A dose of 0·05γ of this material effected a rapid cure of the eye disease in one rat (Fig. 4). These materials would appear to have a higher biological activity than those tested

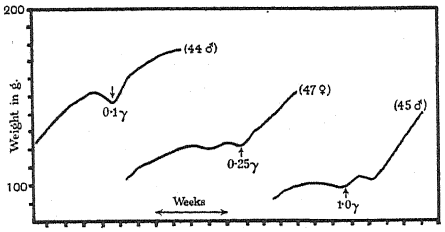


Fig. 3.

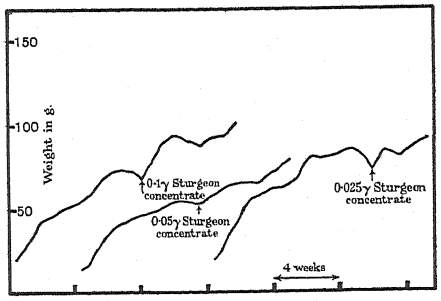


Fig. 4.

Fig. 3. Biological tests on main fraction obtained by distillation of Halibut-liver oil concentrate.
Fig. 4. Showing recovery of growth caused by administration of concentrate of vitamin A prepared from sturgeon-liver oil. Basal diet throughout deficient in vitamin A.

by Euler [1931], Karrer, Morf and Schöpp [1931, 1, 2] and Euler and Karrer [1931], although the differences may to some extent be due to different methods of conducting the feeding experiments.

Discussion.

The position which has now definitely been reached is that the richest products obtained both by us and by Karrer and his collaborators, from mammalian and various fish-livers, are qualitatively and quantitatively indistinguishable in respect of ultra-violet absorption. If the products are not homogeneous, then either the non-vitamin material is relatively diactinic, or the preparations contain substances so closely alike that both exhibit the 328$m\mu$ band. The absorption spectrum affords no conclusive criterion of homogeneity although it would be indeed curious if exactly the same proportion of material exhibiting negligible absorption were present in the products derived from various species of fish. On the other hand, the discovery of isomeric α- and β-carotenes, both transformed *in vivo* into vitamin A-like

substances [Kuhn and Brockmann, 1931] suggests plainly that strict homogeneity must be demonstrated rather than assumed.

If the ultra-violet absorption spectrum leaves the question of purity an open one, the antimony trichloride colour test is equally indecisive. Even if the difficult choice concerning the origin of the 617 and 583 $m\mu$ maxima can be resolved by the demonstration (which is yet to seek) that both bands are traceable to one entity, the existence of the maximum at 693 $m\mu$ and especially (as shown in Table VI) its variable intensity in different preparations, remains wholly unexplained.

Our own observations in general are at least as consistent with Prof. Karrer's tentative structure (I) for vitamin A as with any alternative, and indeed the weight of evidence is impressively though not conclusively in its favour.

From the standpoint of analytical data, the failure to prepare crystalline derivatives of vitamin A makes it very difficult to compel assent to any claims to having prepared the vitamin in a state of purity. The experimental data on molecular weights lead to a value of 320 ± 15 as against 286 on the basis of the formula. In itself, this small discrepancy is not necessarily significant, but when the results of a very large number of ultimate analyses uniformly indicate variable values not quite consistent with the $C_{20}H_{30}O$ formula, doubts concerning purity are strengthened. Finally, our experiments on fully hydrogenated preparations have provided definite evidence of some contaminant in the resulting product.

The analytical data show that the question at issue is not, however, one of gross impurity, and further the extinction coefficient of the 328 $m\mu$ band is of the correct order to be expected from the proposed constitution. In this connection it must be borne in mind that neither C and H percentages nor molecular weights would be affected by heterogeneity arising out of isomeric forms.

As far as can be judged, a small quantity of an alcohol, probably more saturated than vitamin A, is extremely difficult to eliminate completely when reasonably large quantities of material are under investigation. This probably accounts for the failure to reach the highest potency when the full technical resources are applied to cod-liver or poor grade halibut-liver oils.

Once the remaining doubts concerning homogeneity are resolved, biological, spectroscopic and colorimetric standards can be set up in terms of which the results of various methods of assay can be expressed.

We desire to express our thanks to the Department of Scientific and Industrial Research for grants to two of us (R.N.H. and E.T.W.) in aid of this investigation. We are also deeply indebted to Dr J. A. Lovern of the Torry Research Station, Aberdeen, for the great help he has given us in the collection of fish-liver oils, to Messrs Lever Brothers, Ltd., for placing at our disposal samples of mammalian concentrates, to Dr F. H. Carr and Mr W. Jewell of

British Drug Houses, Ltd., for carrying out the vitamin distillations, and to Mr W. Doran and Mr W. F. Boston of Liverpool University for carrying out the numerous micro-analyses recorded in this paper. Part of the expenses of this investigation was defrayed by a grant from the Medical Research Council, for which we express our thanks.

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Note added August 16th, 1932. Since this paper was written we have received (7. vi. 32) a fresh halibut oil the vitamin A content of which was approximately double that of the richest oil (*e* in Table I) previously examined. The following table shows the potencies at various stages in the extraction of the vitamin. Although the original oil was phenomenally rich in vitamin A (estimated at 10 % vitamin) the final product was not appreciably different from the values recorded in Table VI.

	$E^{1\%}_{1\text{cm.}}$				Analysis	
Material	328 $m\mu$	617 $m\mu$	580 $m\mu$	693 $m\mu$	C %	H %
Halibut-liver oil	164	430	260	50	—	—
Non-sap., filtrate	—	4000	2000	410	82·4	10·6
−60°	—	—	—	—	82·7	10·5
Ditto after adsorption						
(i)	—	3950	2100	560	—	—
(ii)	1350	4520	2360	270	83·3	10·7
Distillate, B.P. 136°	1370	4650	2550	450	83·3	11·0

CXL. THE STRUCTURE OF VITAMIN A.

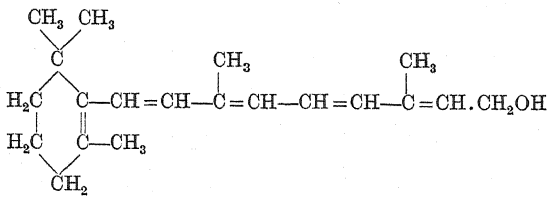
By ISIDOR MORRIS HEILBRON, RICHARD ALAN MORTON and ERIC TAYLOR WEBSTER.

From the Department of Chemistry, University of Liverpool.

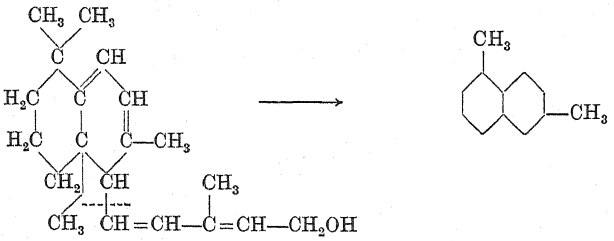
(*Received May 17th, 1932.*)

It has already been shown [Edisbury *et al.*, 1932] that it is possible to obtain by several methods, and especially with acid reagents, a product from vitamin A exhibiting highly characteristic narrow absorption bands. On the basis of the structure (I) suggested by Karrer, Morf and Schöpp [1931] for the vitamin it seemed to us that the new product was probably formed by intramolecular condensation whereby a hydronaphthalene (II) might result.

(I)



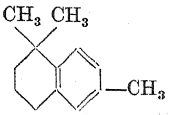
(II)

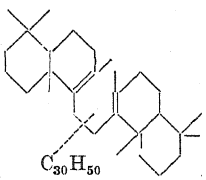


As is well known, many hydronaphthalene compounds can readily be dehydrogenated by sulphur, or better by the Diels method employing selenium, to yield true aromatic naphthalene hydrocarbons. Thus cadalene (1 : 6-dimethyl-4-*iso*propylnaphthalene) or eudalene (1-methyl-7-*iso*propylnaphthalene) is formed from many bicyclic sesquiterpenes [Ruzicka *et al.*, 1922].

On the other hand, ionene (III) [Ruzicka and Rudolf, 1927] and tetra-*cyclo*squalene (IV) [Heilbron and Wilkinson, 1930; Ruzicka *et al.*, 1932], both of which contain *gem*-dimethyl groupings attached to quaternary carbon atoms,

yield on dehydrogenation 1 : 6- and 1 : 2 : 5-trimethylnaphthalene respectively.

(III) 

(IV) 

On the basis of this, we anticipated that the suggested hydronaphthalene derivative (II) would yield with selenium either one or other of the above methylnaphthalenes. On submitting this idea to experimental test we have found that on treatment of the product obtained from a rich concentrate by the action of alcoholic hydrochloric acid with selenium at 300–330°, a good yield of 1 : 6-dimethylnaphthalene results.

The extraordinary ease with which vitamin A is transformed into a cyclised product prompted us to attempt the dehydrogenation with the vitamin itself, with the result that an equally high yield of 1 : 6-dimethylnaphthalene was obtained. It is thus evident that the intramolecular change is due either to the elevated temperature at which the reaction is carried out [cf. Heilbron *et al.*, 1932] or to the action of hydrogen selenide, the presence of which can be observed even at comparatively low temperatures.

Experimental.

A vitamin A concentrate, $E^{1\%}_{1\,cm.}$ 617 $m\mu$, 3140, (7·4 g.), mixed with finely powdered selenium (7·4 g.), was heated under a long air condenser at 300–330° for 48 hours. The cooled viscous product was repeatedly extracted with boiling ether, the combined extracts were filtered and the solvent was removed. The residual oil was then distilled over metallic sodium, whereby the following fractions were obtained.

A. B.P. 120–200°/20 mm. (1·6 g.); redistillation of this fraction over sodium yielded a colourless oil, B.P. 70–110°/5 mm. (1·2 g.).

B. A high boiling fraction, B.P. 200–300°/20 mm. This fraction was obtained as a viscous orange oil which rapidly darkened on standing (1 g.).

1 : 6-*Dimethylnaphthalene.* A warm solution of oil (A) was treated with an alcoholic solution of picric acid (1 g. in 15 cc.), when a slight darkening in colour was immediately observed. On standing the liquid solidified almost completely yielding orange crystals (1 g.) M.P. 100–110°. After recrystallisation from absolute alcohol long orange needles (M.P. 113–114°) were obtained. (Found: C, 56·0; H, 4·2: $C_{18}H_{15}O_7N_3$ requires C, 56·1; H, 3·9 %.) A mixed melting-point with authentic 1 : 6-dimethylnaphthalene picrate (M.P. 112–113°) gave no depression. The hydrocarbon was obtained by shaking an ethereal solution of the picrate with aqueous ammonia. Its absorption spectrum was compared with that of pure 1 : 6-dimethylnaphthalene (Fig. 1) and found to be qualitatively and quantitatively identical.

The *styphnate* was also prepared and forms yellow needles, M.P. 120°. (Found: C, 53·8; H, 3·5. $C_{18}H_{15}O_8N_3$ requires C, 53·9; H, 3·7 %.)

The high boiling fraction (B) failed to give any crystalline derivative with either picric or styphnic acid.

Cyclisation of vitamin A. This was carried out by treating the vitamin A concentrate (11 g.) in alcohol (500 cc.) with concentrated hydrochloric acid (6 cc.). The solution was allowed to stand for 20 minutes at room temperature during which time the pale yellow colour changed to dark brown. Solid sodium carbonate was added until the solution reacted alkaline, when the colour immediately reverted to light yellow. Excess sodium carbonate was filtered off, and the alcohol removed under reduced pressure. The residue was taken up in light petroleum and the petroleum extract repeatedly washed with water. After drying and removal of solvent a light brown viscous oil remained which on spectroscopic examination was found to exhibit well defined absorption bands at 392, 369, 350 and 333 $m\mu$. Treatment of this product with selenium by the method already described above gave 1 : 6-dimethylnaphthalene in a yield of approximately 3·5 %.

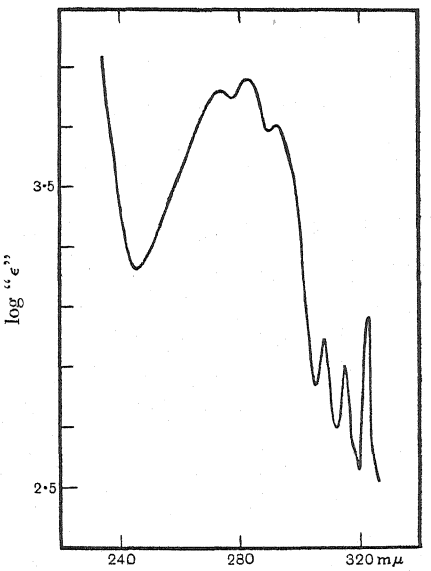


Fig. 1. 1: 6-Dimethylnaphthalene in hexane.

The isolation of 1 : 6-dimethylnaphthalene in good yield by the dehydrogenation of vitamin A definitely establishes the terpenoid nature of the vitamin and the presence in the richest concentrates of a material possessing a constitution which, as far as the fourteenth carbon atom, must be identical with the constitution (I) advanced by Karrer, Morf and Schöpp [1931] for vitamin A.

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CXLI. THE USE OF 7-METHYLINDOLE IN THE ANTIMONY TRICHLORIDE COLOUR TEST FOR VITAMIN A.

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(*Received May 17th, 1932.*)

It is now generally agreed that the blue colour which results when antimony trichloride is added to chloroform solutions of liver oils and concentrates owes its origin, in part at least, to vitamin A. As a quantitative criterion of potency, the test, however, suffers from considerable disadvantages, among which the following may be mentioned.

(*a*) The chromogenic power of the substances present in liver oils is smaller than would be expected from the values obtained with the unsaponifiable extracts [Smith and Hazley, 1930; Smith, 1931; Coward, Dyer, Morton and Gaddum, 1931] owing to the occurrence of inhibitors in the saponifiable portion [*cf.* Norris and Church, 1930, 1, 2]. This effect is highly variable and militates powerfully against accuracy in the colorimetric assay [Lovern, Creed and Morton, 1931; Gillam and Morton, 1931].

(*b*) The coloured solutions exhibit complex and qualitatively variable absorption spectra [Morton, Heilbron and Thompson, 1931; Heilbron, Gillam and Morton, 1931]. Maxima in the spectral absorption curves have been recorded at 572–583, 606–620, 643 and 693 $m\mu$, and there is considerable evidence of complexity in the chromogenic constituents of liver oils.

(*c*) The intensity of absorption at a given wave-length or the absolute depth of blue colour varies considerably with the time elapsing between mixing and recording intensities, and with concentrations in the system: $CHCl_3$—$SbCl_3$—vitamin A—non-chromogenic substances.

It has been shown [Heilbron, Gillam and Morton, 1931] that with many oils the spectral absorption curve shown after reaction with antimony trichloride has occurred consists of a summation of two distinct absorption bands with maxima at 572 and 606 $m\mu$ respectively. Sometimes one, sometimes the other, is the more intense. In all cases the 606 $m\mu$ band eventually becomes the stronger, either by spontaneous ageing or treatment with ozone. Increases in the 606 $m\mu$ band are never at the expense of a reduction in the intensity of the 572 $m\mu$ band, which remains practically constant, as also does the 328 $m\mu$ ultra-violet maximum characteristic of vitamin A. In concentrates, the 606 $m\mu$ band is shifted to 617 $m\mu$ and is always much stronger than the other band. Heilbron, Gillam and Morton [1931] were able to show

that in all cases the shorter wave maximum is present in concentrates at 583 $m\mu$ but that its existence is usually indicated only by an inflexion in the absorption curve. By employing a slight modification of the ordinary procedure in carrying out the colour test they were able to demonstrate the real existence of this maximum. The preliminary addition of a very small quantity of antimony trichloride reagent to the chloroform solution of the concentrate, one minute before carrying out the ordinary test, effects a striking reduction in the intensity of the blue colour. This reduction is largely confined to the absorption band at 617 $m\mu$ so that the 583 $m\mu$ maximum, which is masked in the solution obtained by the ordinary procedure, is now fully revealed.

In discussing the results, the authors envisaged two main possibilities, namely that the two chromogenic mechanisms denoted either two separate chromogens or two distinct reactions between vitamin A and antimony trichloride.

Brode and Magill [1931] also observed the existence of two variable absorption bands in the colour tests, and recorded maxima at 608 and 578 $m\mu$. The solutions were unstable and gradually became red, exhibiting maxima at 532 and 472 $m\mu$. These authors also correlated the 608 and 532 $m\mu$ maxima and considered the latter to arise from a spontaneous decomposition of the substance causing the 608 $m\mu$ band. Similarly the 578 and 472 $m\mu$ maxima were found to be connected in some way. In this laboratory the 532 and 472 $m\mu$ maxima have not been regularly noticed and little evidence of any significant correlation has been obtained. It is, however, important that Brode and Magill obtained valuable evidence of a complex chromogenic mechanism. Like Heilbron, Gillam and Morton [1931] they were however unable to decide whether liver oils contain two separate chromogens or whether the two bands indicate alternative or successive reactions between vitamin A and antimony trichloride.

Another valuable piece of confirmatory evidence was supplied by Emmerie, Eekelen and Wolff [1931]. These authors state: "By treating a vitamin A preparation...with some drops of furan, methylfuran, pyrrol, indol, or skatol (all substances of related chemical structure), and then adding antimony trichloride, the mixture turns purple instead of blue, and in the spectroscope the 610 $m\mu$ band is no longer seen, while the 572 $m\mu$ band remains unaltered. The physiological activity is unimpaired and the preparation (without antimony trichloride) shows the 328 $m\mu$ band just as the original substance did."

Eekelen *et al.* [1931] extended these observations and recorded data which tend to increase the difficulties of interpretation. Apparently, the low values in the colour test for oils are due to the presence of traces of inhibiting substances, but the results, whilst far from conclusive, point rather to heterogeneity of chromogens than to different stages in the reaction between antimony trichloride and vitamin A.

The work of the Dutch investigators seemed to indicate a more convenient method for separating the chromogenic effects than the original one emanating

from these laboratories. Accordingly the statements made were tested and fully substantiated.

A large number of pure substances of the type suggested were tried, including 2-, 3- and 7-methylindole. The most convenient substance was found to be 7-methylindole.

In Table I the fact is demonstrated that the 617 $m\mu$ maximum suffers considerable inhibition before the 583 $m\mu$ maximum is appreciably affected. In

Table I. *Effect of 7-methylindole on the antimony trichloride colour test applied to materials very rich in vitamin A.*

	Wt. of concentrate used mg.	Wt. of 7-methyl-indole used mg.	$E^{1\%}_{1\,cm.}$ 617 $m\mu$	$E^{1\%}_{1\,cm.}$ 583 $m\mu$	Ratio wt. 7-methyl-indole/wt. concentrate
1	0·0562	0·375	660	1070	6·5
	,,	0·300	750	1100	5·2
	,,	0·225	910	1180	3·9
	,,	0·150	1250	1250	2·6
	,,	0·075	1525	1310	1·3
	,,	0	2350	1310	—
	,,	Trace only	—	1300	—
2	0·0522	0·375	690	965	7·18
	,,	0·3	790	960	5·76
	,,	0·225	1206	1200	4·3
	,,	0·150	1300	1210	2·88
	,,	0·075	1870	1260	1·44
	,,	0	2160	1350	—
3	0·05	0	3300	1600	—
	,,	0·2	1600	1550	4
4	0·04	0	4200	2135	—
	,,	0·16	2000	1980	4

Table II the last column indicates the intensity at 583 $m\mu$ after just sufficient 7-methylindole has been added to render the 617 $m\mu$ maximum definitely weaker than the 583 $m\mu$ maximum. The main results may be summarised as follows.

(1) 7-Methylindole, at the concentrations used, is practically transparent in the region 290–350 $m\mu$.

(2) The absorption spectrum of a solution containing vitamin A concentrate and 7-methylindole in chloroform agrees quantitatively with the summation of the separate curves for the concentrate and the 7-methylindole, so that there is no evidence of chemical interaction before the addition of antimony trichloride.

(3) In all cases, from cod-liver oils to the richest concentrate, the incorporation of a trace of 7-methylindole effects an inhibition of the capacity to give the blue colour.

(4) The inhibition can be regulated by adding greater or smaller quantities of 7-methylindole.

(5) The masking effect of the 617 band on the 583 $m\mu$ maximum can be eliminated by adding a carefully regulated quantity of inhibitor so that the

Table II.

Material	$E^{1\%}_{1\,cm.}$ 328 $m\mu$ Ultra-violet absorption	$E^{1\%}_{1\,cm.}$ 617 $m\mu$ Antimony trichloride colour test without added 7-methylindole	$E^{1\%}_{1\,cm.}$ 583 $m\mu$ Antimony trichloride colour test without added 7-methylindole	$E^{1\%}_{1\,cm.}$ 583 $m\mu$ (colour test) intensity when just enough 7-methylindole has been added to make 617 $m\mu$ band weaker than that at 583 $m\mu$
Halibut-liver oil non-sap.	1000	2350	1350	1300
(average)	1154	3030	1450	1300
Pig-liver concentrate	525	1200	840	650
Cod-liver oil concentrate	270	541	360	315
Mammalian concentrate	130 *(290, 190)	320	230	200
Cod-liver non-sap.	115	221	124	110
,, ,,	57·5	96	65	60
Pig-liver oil (*a*)	30	63	41	34
,, (*b*)	14·4	36·8	17·5	14·8
Pig-liver oil (store pigs) (*a*)	—	0·99	0·92	0·9
,, ,, (*b*)	—	1·6	1·1	1·0
Cod-liver oil crude non-sap.	16 *(290, 14)	34·4	18	16
Sheep-liver oil	19·7 *(290, 25·7)	30	28	28
Cod-liver oil	0·73	0·76	0·59	0·5
,,	5·5	15·2	9·0	8·2

* Occasionally in fish-liver oils and concentrates, and more often in materials from mammalian livers, we have observed an absorption band (max. 290 $m\mu$) superposed on the 328 $m\mu$ band of vitamin A. Dr T. Moore and Dr N. S. Capper have also observed this effect (private communication). At first sight this band would seem to be due to the decomposition product with a maximum at 290 $m\mu$. This explanation is not wholly satisfactory because the materials exhibiting a maximum at 290 $m\mu$ without other evidence of decomposition, exhibit high values in the colour test for the 583 $m\mu$ band. A substance closely related to vitamin A and not derived from it by decomposition may be responsible. In this connection it is well to emphasise a point which merits further investigation: as has been shown by Kuhn and Brockmann [1931], α-carotene and β-carotene, containing α- and β-ionone rings respectively, are both capable of inducing growth. Presumably each is converted *in vivo* into a vitamin A-like substance. There is thus the possibility of two isomeric vitamin A compounds, one with and one without the ethenoid linkage of the ring conjugated to the side chain. The properties of such isomerides cannot be predicted but it is quite possible that the 290 and 328 $m\mu$ bands may respectively be due to the α- and β-isomerides.

617 and 583 $m\mu$ bands can be seen side by side, at approximately equal intensities, before any appreciable inhibition of the 583 $m\mu$ band has occurred.

(6) Using the richest vitamin A concentrates (Carr-Price blue value $> 50{,}000$), the 617 $m\mu$ band is almost exactly twice as intense as the 583 $m\mu$ maximum without addition of inhibitor, but if 7-methylindole is added before the antimony trichloride reagent, in the ratio 7-methylindole/concentrate = 4/1, the two bands appear with roughly equal intensity, the inhibition being confined to the longer wave maximum.

(7) The addition of larger quantities of 7-methylindole effects a slow inhibition of the 583 $m\mu$ band, but this is always very much smaller than the inhibition of the 617 $m\mu$ maximum.

(8) In carrying out the antimony trichloride colour test as a quantitative measure of vitamin A potency, serious inaccuracies arise when the test is carried out on oils owing to the variable action of inhibitors, but these are greatly reduced when observations are carried out on the non-saponifiable

matter [Smith and Hazley, 1930; Coward *et al.*, 1931]. In all cases, much more accurate results can be obtained if attention is concentrated on the 583 $m\mu$ maximum, using just sufficient 7-methylindole to allow the 617 and 583 $m\mu$ bands to be seen in the spectroscope. Under these circumstances, the estimates of potency are in close agreement with the values deduced from the intensity of the 328 $m\mu$ band.

(9) Accepting the molecular weight of vitamin A as near 300 (7-methylindole, 133) and making use of the fact that four parts by weight of inhibitor to one of the richest vitamin preparations are necessary for optimum results, this is equivalent to approximately 10 molecules of 7-methylindole to 1 mol. vitamin.

(10) Inhibition by means of 7-methylindole does not readily occur with the 693 $m\mu$ band recorded by Morton, Heilbron and Thompson [1931], and it does not appear to be possible to establish any rigid correlation between this band and the 583 $m\mu$ maximum.

(11) In view of the fact that inhibition occurs with complete success when the colour tests on the richest concentrates of vitamin A are carried out in the presence of 7-methylindole, such concentrates cannot be accepted as homogeneous without a satisfactory explanation of the apparent independence of the 617 and 583 $m\mu$ bands. Vitamin A may well react in two stages with antimony trichloride, but there is no direct evidence on this point. The ultraviolet absorption spectrum discloses definite information of only one absorbing entity, the colour test reveals two chromogenic mechanisms. No decision is yet possible as to whether the richest concentrates are mixtures of extremely closely related substances, or whether one and the same molecule can give rise to the complex colour reaction.

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CXLII. THE IMMUNOLOGICAL PROPERTIES OF ALKALI-TREATED PROTEINS.

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The influence of acid and alkali on proteins in relation to the antigenic properties of these proteins has been studied by many authors. Wells [1909] noted the marked loss of antigenic power when crystalline egg-albumin was treated with sodium hydroxide, whereas little loss occurred following similar treatment with hydrochloric acid. Ten Broeck [1914] found that racemised egg-albumin (prepared by Dakin's [1912] method) was non-antigenic, and similar results with other proteins were obtained by Landsteiner and Barron [1917] and by Kahn and McNeal [1918].

Evidence of restoration of antigenic properties to alkali-treated proteins was furnished by Landsteiner and Barron [1917], who showed that when the non-antigenic alkali-treated serum-proteins were treated with nitric acid, antigenic xanthoproteins were formed; these xanthoproteins prepared from alkali-treated proteins gave precipitin and complement-fixation reactions with antisera to xanthoproteins from untreated serum-proteins and also acted as true antigens when injected into rabbits. These results, indicating some reversal of the process or processes which originally caused loss of antigenic power, are very significant, and it is possible that a further study of this phenomenon would add considerably to our knowledge of protein antigens.

In view of the importance of this relationship between the action of alkali and the loss of antigenic properties, it was decided to attempt to define more precisely the relationship between p_H and loss of antigenicity, and also to try to obtain, by some method other than that used by Landsteiner and Barron [1917], confirmatory evidence of the restoration of antigenic power to alkali-treated proteins. For the latter purpose, it was considered that iodination of the alkali-treated proteins would be most suitable, since iodination can be effected in neutral or faintly alkaline solution, and, although it replaces the original species specificity of serum proteins by a specificity determined principally by the 3 : 5-diiodotyrosine groupings [Wormall, 1930], iodination does not lead to any appreciable loss of antigenic properties.

In the investigation described here, the proteins of horse-serum were rendered non-antigenic by treatment with sodium hydroxide for approximately 24 hours at 18° in one series and 30° in another series. Fractions of these "alkali-proteins" were nitrated and others iodinated, and precipitin

and complement-fixation tests were then carried out with these preparations to determine whether the power to react with various antisera had been restored by nitration and iodination. These protein preparations, the alkali-treated proteins and the corresponding iodinated and nitrated alkali-treated proteins, were also tested for antigenic power by injection into rabbits, precipitin and complement-fixation tests being made at intervals.

Experimental.

Antigens used for injection.

The following antigens were prepared for immunisation purposes. NaCl to give 0·9 %, and phenol to give 0·25 % were added to all the antigen solutions, except those containing iodoprotein, which received NaCl only. All the solutions were kept in the ice-chest.

NaOH-treated serum-proteins. (*a*) *At* 18°. A mixture of 1500 cc. of horse-serum, 235 cc. of 7·4 *N* NaOH and 15 cc. of toluene was kept at 18° for 22 hrs. Conc. HCl was added to give maximum precipitation, and the precipitated proteins were filtered off and washed well with water; they were then dissolved in water with the addition of a small amount of *N*/2 NaOH and sufficient 10 % Na_2CO_3 solution to give a clear solution with p_H about 7·8. NaCl and phenol were then added in the amounts given above and the solution kept in the ice-chest. The final volume was 1000 cc. and the solution contained 5·75 % of protein (solution *A*).

(*b*) *At* 30°. A mixture of 2500 cc. of horse-serum, 225 cc. of 12·15 *N* NaOH and 25 cc. of toluene was kept in an incubator at 30° for 24 hrs. The proteins were precipitated by HCl as above, and finally obtained in solution (800 cc.) at about p_H 7·8. The solution contained 5·6 % of protein (solution *B*).

Iodinated NaOH-treated proteins. (*a*) 250 cc. of the solution of NaOH-treated proteins prepared as above (solution *A*) were mixed with 250 cc. of distilled water and 250 cc. of 7 % NH_4OH solution, and treated with *N*/10 iodine solution until there was a considerable excess of iodine [*cf.* Wormall, 1930]. The iodinated proteins were precipitated by the addition of 10 % acetic acid to give maximum precipitation, and the precipitated protein was removed by centrifuging. This precipitate was washed with water and dissolved in water with the addition of sufficient 10 % Na_2CO_3 solution to give a clear solution, the reaction of the solution being adjusted to p_H 7·6. 250 cc. of a solution containing 5·2 % of protein were obtained in this manner.

(*b*) 250 cc. of the solution obtained by treating horse-serum with NaOH at 30° (solution *B*) were iodinated as above.

Nitrated NaOH-treated proteins (*xanthoproteins*). The method used for nitration was that involving the action of conc. HNO_3 at 18° in the presence of urea [*cf.* Landsteiner and Prášek, 1913]. The products of this reaction react immunologically like proteins nitrated by a method which does not involve treatment with strong acid, *viz.* the tetranitromethane method [Wormall, 1930], and in this paper the terms xanthoprotein and nitrated protein are used synonymously.

(*a*) To 300 cc. of a solution of NaOH-treated proteins (solution *A*) a few grams of urea were added followed by 600 cc. of conc. HNO_3 slowly with constant stirring and cooling. The mixture was kept for 45 mins. at 18° and then poured into a large volume of distilled water. The precipitate was then filtered off, washed with water and dissolved in water with the addition of a little *N*/5 NaOH and sufficient 10 % Na_2CO_3 solution to give a clear solution. The p_H was adjusted to 7·8 and 300 cc. of a solution containing 3·5 % of protein were obtained.

(*b*) 300 cc. of the protein solution obtained by NaOH-treatment at 30° (solution *B*) were nitrated in a similar manner.

Immunisation.

Each antigen solution was tested on four rabbits, the injections being made weekly. Serum from each rabbit was tested 7 days after the third and each subsequent injection, *i.e.* just before the fourth, fifth, sixth and seventh, and, in certain instances, the eighth injection, and 7 days after the final injection.

Series (*a*). For the first three injections, an amount of the solution (solution A or the corresponding iodinated or nitrated protein solution) which contained approximately 0·25 g. of protein (4·3 cc. to 7 cc.) was injected intravenously into each rabbit. Five further injections of a volume of the solution which contained approximately 0·35 g. of protein were made intraperitoneally.

Series (*b*). With solution B and the corresponding iodinated and nitrated protein solutions each rabbit received at each injection a volume of the solution which contained 0·25 g. of protein; each animal had seven injections, the first three or four being intravenous and the rest intraperitoneal.

Precipitin tests.

Two, and, in a few tests, three drops of the immune serum were added to 0·25 cc. of the diluted antigen solution in a small test-tube ($2'' \times \frac{1}{4}''$). The mixture was shaken and observed after it had stood 5 mins., 30 mins., 1 hr. and 3 hrs. at room temp. (16–20°) and after a further 18 hrs. in the ice-chest. The amount of protein in each antigen solution had previously been determined, and the various antigen dilutions were made to correspond to the following dilutions of a 5 % protein solution; 1 : 20, 1 : 100, 1 : 500 and 1 : 2500. Several immune sera were used for each series of the tests, but since similar antisera gave essentially the same results, the reactions in each instance with one typical antiserum only are recorded in the Tables of this paper. The readings, unless otherwise stated, are those obtained after 1 hour.

The extent of precipitin formation is recorded as follows: – (no reaction), f.tr. (faint trace), tr. (trace), ±, +, +±, ++ (increasing degrees of precipitation).

Complement-fixation tests.

A range of the antigen solution was made in a series of small test-tubes, each tube containing 0·5 cc. of the diluted antigen; 0·06 cc. of 1 : 5 guinea-pig-serum and 0·10 cc. of the inactivated rabbit-"immune" serum were added to each tube, the tubes shaken and allowed to stand for 45 mins. to 1 hr. 0·5 cc. of a 4 % suspension of sensitised ox-red blood cells was then added to each tube, the tubes shaken and incubated at 37°. The amount of haemolysis after various intervals, usually 15 mins., 30 mins. and 1 hr., was recorded. On some occasions similar series of tests were carried out with 0·05 and 0·15 cc. of immune serum and varying concentrations of antigen. Control tests with no antigen solution, and others with varying amounts of antigen and no immune serum, were always carried out.

The influence of p_H *on the power of horse-serum proteins to react with antibodies to unchanged horse-serum.*

In these experiments samples of normal horse-serum were kept at various hydrogen ion concentrations for 24 hrs. at 19°, and the power of such treated sera to react with antisera to untreated horse-serum was tested at intervals.

The adjustment of the horse-serum to a definite p_H was made by the addition of N HCl or N NaOH, and to the solution obtained a buffer solution (the universal buffer mixture of Prideaux and Ward [1924] (B.D.H. preparation) of the same p_H was added, the presence of this buffer mixture preventing any appreciable alteration in the p_H of each solution during the course of the experiment. For the solutions to be kept at p_H 1, N HCl was added to the diluted serum to give the desired p_H, and the solution then diluted with $N/10$ HCl; N and $N/10$ NaOH were used in the same way for the tests at p_H 13.

In the experiment recorded in Table I, 6 cc. of normal horse-serum were diluted with about 20 cc. of 0·9 % NaCl, and N HCl or N NaOH was added until the desired p_H was obtained; 20 cc. of universal buffer mixture of the corresponding p_H were added, and the mixture diluted to 60 cc. with 0·9 % NaCl. These mixtures were then kept at 19°, and samples withdrawn from each solution at the start, after 45 mins., 3½ hrs. and 24 hrs. Each sample, except those from the solution at p_H 7·5, was neutralised by the addition of acid or alkali and the reaction adjusted to p_H 7·5, after which the required dilutions were made and precipitin tests carried out. In Table I the results with p_H 4, 5, 6, 7, 8, 9 and 10 are omitted, since these did not differ appreciably from those given for p_H 7·5.

Table I. *The influence of* p_H *on the power of horse-serum-proteins to react with antibodies to untreated horse-serum-proteins.*

Tests carried out with anti-horse-serum immune serum No. 107 (readings after 1 hr.).

Duration of treatment at different p_H values	Precipitin reactions with horse-serum-proteins kept for various periods at the following p_H and subsequently neutralised							Dilution* of antigen after neutralisation
	1	2	3	7·5	11	12	13	
Start	+ ±	+ ±	+ ±	+ ±	+ ±	+ ±	+ ±	1 : 20
	+ +	+ +	+ +	+ +	+ +	+ +	+ +	1 : 100
	+	+	+	+	+	+	+	1 : 500
	tr.	tr.	tr.	tr.	tr.	tr.	tr.	1 : 2500
45 mins.	+ ±	+ ±	+ ±	+ ±	+ ±	+	±	1 : 20
	+ ±	+ +	+ +	+ +	+ +	+ +	+	1 : 100
	±	±	+	+	+	±	tr.	1 : 500
	f.tr.	f.tr.	tr.	tr.	tr.	tr.	f.tr.	1 : 2500
3½ hrs.	+	+ ±	+ ±	+ ±	+	+	tr.	1 : 20
	+ ±	+ +	+ +	+ +	+ ±	+	±	1 : 100
	±	±	+	+	+	±	tr.	1 : 500
	tr.	tr.	tr.	tr.	f.tr.	–	–	1 : 2500
24 hrs.	+	+ ±	+ ±	+ ±	±	±	–	1 : 20
	+ ±	+ +	+ +	+ +	+ +	±	–	1 : 100
	±	±	+	+	+	tr.	–	1 : 500
	f.tr.	f.tr.	f.tr.	tr.	tr.	–	–	1 : 2500

* All antigen dilutions in this and in the following Tables are in terms of dilution of a 5 % protein solution.

The results of these experiments confirm the findings of the earlier workers in that they show marked destruction of antigenicity in alkaline solution and a less pronounced loss by treatment with acid. This investigation of the exact influence of p_H, rather than the study of the action of excess of strong acid

or alkali, leads to the conclusion, however, that in moderately alkaline solutions the loss of the power to give precipitation with antibodies to the unchanged serum is not as rapid as some authors have suggested. Thus, horse-serum kept at p_H 11 for 24 hrs. at 19° shows a slight loss only of this antigenic power. At p_H 12, however, marked loss occurs in $3\frac{1}{2}$ hrs. and at p_H 13 a similar destruction occurs in less than 1 hr.; after 24 hrs. at p_H 13, the serum no longer possesses the power to react with antisera to the native proteins of horse-serum.

In some of the above, and in later experiments, antisera obtained by the injection into rabbits of fat- and lipin-free proteins of normal horse-serum, obtained by the method used by Hartley [1925], were used. In all these experiments, antisera obtained in this way gave results identical with those given by antisera obtained by the injection of untreated horse-serum.

The action of strong alkali on the power of serum-proteins to react with antibodies to the unchanged serum-proteins and the immunological properties of these alkali-treated proteins after iodination and nitration.

Preliminary tests were carried out to determine the amount of NaOH necessary for the destruction, in 20–24 hrs. and at various temperatures (18°, 30° and 37°), of the power of horse-serum-proteins to react with antisera to untreated horse-serum-proteins. After the mixtures had stood for this length of time at the required temperature, conc. HCl was added to give maximum precipitation and the precipitated proteins were centrifuged and dissolved in 0·9 % NaCl solution with addition of the minimum amount of 10 % sodium carbonate solution to give a clear solution. Portions of these solutions of alkali-treated proteins were iodinated or nitrated by the methods indicated above, and the products obtained tested with various antisera. In the majority of these experiments it was found that, although sufficient strong alkali had been added to render the proteins incapable of reacting (in precipitin and complement-fixation tests) with antibodies to untreated horse-serum-proteins, the proteins formed could still take part in immunological reactions after iodination or nitration; thus, after iodination they gave reactions with antisera to iodinated horse-serum-proteins, and after nitration they reacted with antisera to nitrated horse-serum-proteins, the latter observation confirming those of Landsteiner and Barron [1917]. The iodinated alkali-treated proteins were not, however, always as strongly antigenic *in vitro* as were the corresponding nitrated proteins, but usually, after iodination or nitration, these "alkali-proteins" gave good precipitin reactions. In other experiments, where the amount of NaOH was much greater than in the experiments described here, no regeneration of antigenic properties by iodination or nitration could be effected, but this is not surprising in view of the pronounced hydrolytic and other destructive changes which occur when serum-proteins are treated with strong alkali. Table II records an experiment which illustrates some of these features. This experiment, involving the action of NaOH at 37°, shows that

on occasions the antigenic powers *in vitro* can best be restored with HNO_3, for here the nitrated proteins of solution *Y* (a solution of alkali-treated horse-serum-proteins) gave good precipitin reactions with suitable antisera, whereas little precipitation was obtained with iodinated proteins of solution *Y* and antisera to iodinated horse-serum-proteins. In the case of solution *Z*, where much more alkali was used, no appreciable regeneration of the power to give precipitin reactions could be effected by iodination or nitration.

Table II. *Precipitin reactions.*

Antigen	Antigen dilution	Immune serum: Anti-horse-serum (No. 107)	Immune serum: Anti-iodo-horse-serum (No. 1657)	Immune serum: Anti-nitro-horse-serum (No. 1881)
Horse-serum (*X*)	1 : 100	++	–	f.tr.
	1 : 500	+±	tr.	tr.
	1 : 2500	+	tr.	f.tr.
NaOH-treated horse-serum (*Y*)	1 : 100	–	–	–
	1 : 500	–	–	–
	1 : 2500	–	–	–
NaOH-treated horse-serum (*Z*)	1 : 100	–	–	–
	1 : 500	–	–	–
	1 : 2500	–	–	–
Iodinated *X*	1 : 100	tr.	++	tr.
	1 : 500	tr.	±	±
	1 : 2500	–	tr.	tr.
Iodinated *Y*	1 : 100	–	±	–
	1 : 500	–	tr.	–
	1 : 2500	–	–	–
Iodinated *Z*	1 : 100	–	f.tr.	–
	1 : 500	–	–	–
	1 : 2500	–	–	–
Nitrated *X*	1 : 100	–	–	f.tr.
	1 : 500	–	–	+
	1 : 2500	–	–	±
Nitrated *Y*	1 : 100	–	–	+
	1 : 500	–	–	±
	1 : 2500	–	–	f.tr.
Nitrated *Z*	1 : 100	–	–	f.tr.
	1 : 500	–	–	tr.
	1 : 2500	–	–	–

Experimental details.

X. 30 cc. of horse-serum + 1 cc. of toluene.
Y. „ „ + 6·9 cc. of 5·35 *N* NaOH + 1 cc. of toluene.
Z. „ „ + 10·5 cc. „ + „

These mixtures were kept at 37° for 20 hrs. The "alkali-proteins" of solutions *Y* and *Z* were precipitated by HCl and dissolved as described for the antigens used for injection. The amount of protein in each solution was determined and the antigen dilutions were calculated to refer to 5 % protein solutions.

Table III gives the results of precipitin tests with some of the antigens used for the injection experiments (series (*a*)), and here the restoration of *in vitro* antigenic power to alkali-treated proteins appears to be effected just as readily by iodination as by nitration. Results of a similar nature were obtained in complement fixation tests. The iodinated alkali-treated proteins also reacted with antisera to brominated horse-serum-proteins.

Table III. *Precipitin reactions of the antigens used for the injections (series (a)).*

Antigen	Antigen dilution	Immune serum: Anti-horse-serum (No. 107)	Anti-iodo-horse-serum (No. 1657)	Anti-bromo-horse-serum (No. 1822)	Anti-nitro-horse-serum (No. 1881)
NaOH-treated horse-serum-proteins (*a*)	1 : 20	–	–	–	–
	1 : 100	–	–	–	–
	1 : 500	–	–	–	–
	1 : 2500	–	–	–	–
Iodinated NaOH-treated horse-serum-proteins (*a*)	1 : 20	–	–	tr.	–
	1 : 100	–	+ ±	+	tr.
	1 : 500	–	+	±	f.tr.
	1 : 2500	–	f.tr.	f.tr.	–
Nitrated NaOH-treated horse-serum-proteins (*a*)	1 : 20	–	–	tr.	±
	1 : 100	–	–	tr.	+
	1 : 500	–	–	–	±
	1 : 2500	–	–	–	tr.
Horse-serum	1 : 20	+ +	–	–	–
	1 : 100	+ +	tr.	–	f.tr.
	1 : 500	+	tr.	–	tr.
	1 : 2500	±	f.tr.	–	f.tr.

The in vivo *antigenic properties of alkali-treated proteins and of alkali-treated proteins which have been iodinated or nitrated.*

The results of the experiments described above show that proteins which have been treated with sufficient alkali to destroy the power to react with antibodies to the original protein have not necessarily lost their antigenic capabilities, and that they can function as *in vitro* antigens after iodination or nitration. From these test-tube experiments it would not be justifiable to conclude, however, without further evidence, that restoration of antigenic properties had occurred, for several other possible explanations could be presented. The action of the alkali might, for example, result in the formation of denatured proteins with a new specificity, though such denatured proteins would be expected to give some reaction with antisera to the original protein. The most conclusive evidence would undoubtedly be furnished by immunisation experiments if they showed that, whereas no antibodies are produced in the animal body in response to the alkali-treated proteins, antibodies are readily formed after the injection of the corresponding iodinated or nitrated alkali-treated proteins.

The experiments to be referred to under this heading can be divided into two groups: series (*a*), in which were used horse-serum-proteins which had been treated with sufficient sodium hydroxide solution to give a final concentration approximately N and the mixture kept for 22 hrs. at 18°, and series (*b*) with a somewhat similar mixture kept at 30° for 24 hrs. Portions of each solution of alkali-treated proteins were iodinated and others converted into xanthoproteins, and immunisation experiments carried out with the "alkali-protein" and the corresponding iodinated and nitrated proteins. Four rabbits

were used for each antigen solution, and the serum of each animal was tested frequently, in precipitin and complement fixation tests, against the antigen solution used for the injections and against certain other antigens. Each rabbit received seven or eight weekly injections, and the serum tests were made 7 days after the third and subsequent injections. The results of these experiments supported, in general, the conclusion drawn from the results of the experiments already described above, the experiments with the xantho-proteins prepared from the "alkali-proteins" confirming the findings of Landsteiner and Barron [1917]. The antibody response to the iodinated and nitrated compounds, particularly to the former, was perhaps not as marked as was expected, but possibly this can be accounted for by the more drastic treatment with alkali. In these experiments the concentration of sodium hydroxide was approximately the same, but the incubation period was longer than in the experiments of Landsteiner and Barron (22 and 24 hrs. compared with 16 hrs.) and in series (*b*), at least, the incubation temperature was higher. The mixtures prepared by the latter authors were kept at room temperature, whereas those used in the experiments described here were kept at 18° (series (*a*)) and 30° (series (*b*)).

Table IV. *Precipitin reactions between the antigens used for the injections (series (a)) and the sera of the rabbits injected.*

Rabbit No.	Rabbit injected with	Antigen used in test	A Antigen dilution 1:20	1:100	1:500	1:2500	B Antigen dilution 1:20	1:100	1:500	1:2500
120	NaOH-treated serum-proteins (*a*)	NaOH-treated serum-proteins (*a*)	–	tr.	tr.	–	tr.	tr.	f.tr.	–
121			–	–	–	–	–	–	–	–
122			–	tr.	tr.	–	–	tr.	f.tr.	–
123			–	–	–	–	–	–	–	–
124	Iodinated	Iodinated	–	–	–	–	f.tr.	tr.	tr.	–
125	NaOH-treated serum-proteins	NaOH-treated serum-proteins	f.tr.	±	±	f.tr.	tr.	+	±	–
126			–	–	–	–	–	–	–	–
127			–	tr.	f.tr.	–	tr.	±	f.tr.	–
128	Nitrated	Nitrated	tr.	±	±	f.tr.	±	±	tr.	f.tr.
129	NaOH-treated serum-proteins	NaOH-treated serum-proteins	f.tr.	±	±	f.tr.	tr.	±	tr.	–
130			tr.	+	±	f.tr.	+	+	±	f.tr.
131			tr.	tr.	f.tr.	–	f.tr.	tr.	–	–

In the above table, A refers to tests made with sera of the rabbits after these animals had received three injections of the antigen, and under B are given the results with the sera obtained after five injections.

Table IV gives some typical results of the precipitin tests with the sera of the rabbits of series (*a*), and Table V some similar complement-fixation tests. The most pronounced differences are found when the NaOH-treated proteins and the corresponding nitrated compounds are compared. The former engender practically no antibody response, whereas the injection of the latter causes the production of antibodies for the antigen used. The iodinated alkali-treated proteins give some antibody response, and, although it is not as marked as that with the corresponding nitrated compounds, it is not insignificant.

Table V. *Complement-fixation tests with each antigen used for the injections (series (a)) and the sera of the rabbits injected with this antigen.*

Rabbit No.	Proteins used as antigen in test and for immunisation	Antigen dilution used 1:20	1:80	1:320	1:1280	1:2560	Control (NaCl)
120	NaOH-treated serum-proteins	4	4	3	3	4	4
121		4	4	4	4	4	4
122		4	2	1	–	1	4
123		4	4	4	4	4	4
124	Iodinated NaOH-treated serum-proteins	4	4	4	–	1	4
125		4	3	–	–	–	4
126		3	2	1	–	–	4
127		2	1	–	–	–	4
128	Nitrated NaOH-treated serum-proteins	4	2	–	–	–	4
129		2	–	–	–	–	4
30		2	–	–	–	–	4
131		3	2	–	–	1	4

Note. Control tests indicated that the antigen solutions, in the concentrations used here, had neither anticomplementary nor haemolytic powers.

In the above Table, 4 indicates complete haemolysis, – indicates no haemolysis, and 3, 2 and 1 give intermediate degrees of haemolysis.

The results with the proteins of series (*b*) (alkali-treatment at 30°) were similar to those in Tables IV and V, with somewhat less marked precipitin and complement-fixation reactions. Treatment of serum-proteins with strong alkali at this higher temperature probably results in such pronounced hydrolytic and other changes that regeneration, even in part, of antigenic properties is no longer possible. The nitrated proteins of this series, however, did give some antibody response, and marked complement-fixation reactions were obtained. The sera of the rabbits injected with iodinated proteins of this series gave no significant precipitin reactions, but did give some complement-fixation when tested against the corresponding antigen. Thus, in this series, the only satisfactory positive results were obtained in complement-fixation tests.

The sera of all these animals were tested on certain occasions with similar proteins which had not been subjected to the action of strong alkali, *viz.* with horse-serum-proteins, and also with iodinated and nitrated horse-serum-proteins. The results were similar to those described above and recorded in Tables IV and V, with, as was expected, more pronounced precipitation or complement fixation. Thus, the sera of rabbits injected with iodinated alkali-treated proteins reacted with iodinated horse-serum-proteins, but not with nitrated horse-serum-proteins or with untreated horse-serum-proteins, and the sera of rabbits injected with nitrated alkali-treated proteins reacted with the nitrated serum-proteins only.

Discussion.

The destructive action of alkali on protein antigens has long been recognised [Wells, 1909, *etc.*] and several explanations have been put forward to account for this loss of antigenicity. Some loss of this function does occur during similar treatment with acid of approximately the same strength, but this action of acid is very mild compared with that of alkali. Alkalis appear

to have, therefore, a somewhat specific destructive action on protein antigens, and this action does not seem to be related to hydrolytic changes effected in the protein molecule.

The immunological properties of horse-serum-proteins which have been kept in buffered solutions of known p_H for varying lengths of time were first investigated in this work, with results which are of some value in relation to the general study of the immunology of chemically altered protein antigens. As was expected, destruction of the power of such proteins to react with antibodies to the untreated proteins occurred most readily in alkaline solution, but it was rather remarkable that there was no appreciable loss of this function after exposure for 24 hrs. (at 19°) to alkaline solutions at or below p_H 11. At p_H 12 there was moderate, and at p_H 13 marked, loss of this power in less than 1 hr. In acid solutions of p_H 1 and 2 there was only a very slight diminution in 24 hrs. at 19° of the power to give a precipitate with antisera to untreated horse-serum-proteins, and no detectable loss occurred in the solutions kept for this period at p_H 3–10 inclusive. The serum-proteins are certainly not the least sensitive of proteins to treatment with acid or alkali, and thus it appears rather remarkable that such changes in the reaction of the medium can occur without appreciable loss of these immunological properties. Any prolonged exposure of proteins to relatively strongly acid or alkaline solutions will undoubtedly cause serious alteration of the immunological behaviour of these proteins, but from the experiments recorded here it appears justifiable to conclude that exposure for short periods and at the ordinary temperatures to reactions between p_H 2 and p_H 11 will not appreciably alter the protein antigens of serum. In studying the immunological properties of chemically altered protein antigens therefore, although it is always advisable to avoid, where possible, the use of strong acid or alkali, especially the latter, the use of reagents of this type must not necessarily be condemned.

The loss of antigenic properties as a result of the action of alkali might be attributed to any one, or to a combination of two or more, of the changes which occur in the protein molecule. Hydrolysis, racemisation and changes in the sulphur-containing constituents of the protein take place in the alkaline medium, and in all probability there are other ways in which the protein molecule is modified by such treatment. Most authors consider that the loss of antigenicity is related to racemisation. For a discussion of the evidence see Wells [1929] and Hartley [1931]. Hartley suggests, however, that antigenicity is lost before racemisation is complete. No attempt has been made in the work described here to study this relationship or indeed to attempt to relate loss of antigenicity with any definite change or changes in the protein molecule, and the main problem was concerned with the possibility of reversing this destructive action of sodium hydroxide. Regeneration of antigenic power in alkali-treated serum-proteins can be effected by treatment with conc. nitric acid [Landsteiner and Barron, 1917], and the experiments described here have fully confirmed this observation.

The other method used in these attempts to obtain antigenic preparations from non-antigenic alkali-treated serum-proteins involved iodination. Iodination of proteins leads to no loss of antigenic power, but the original species specificity is lost and a new specificity acquired [Obermayer and Pick, 1906], this new specificity being characteristic for the 3 : 5-dihalogenated tyrosine grouping [Wormall, 1930]. Iodination of the alkali-treated proteins used in this work led to some regeneration of antigenic function (*cf.* Tables II, III, IV and V); thus the iodinated alkali-treated proteins reacted *in vitro* with antisera to iodoproteins from horse-serum, and after injection into rabbits they produced characteristic iodoprotein antibodies. The antigenic power regenerated was, however, not very high and certainly was not as pronounced as that released by nitration of the same alkali-treated proteins. It is not to be expected, however, that iodination or nitration will lead to complete regeneration of antigenic power, and the margin between temporary loss (which perhaps is reversed in part by nitration or iodination) and irreversible destruction of antigenicity will probably be small. This loss of antigenicity is probably due to several changes in the protein molecule, and nitric acid (or iodine in ammoniacal solution) may be able to reverse certain changes only. Whatever the reason for the regeneration of antigenic properties, it will probably be concerned with the tyrosine groupings of the protein molecule, groupings which are affected by nitration and iodination, and these findings emphasise still further the significance, from the immunological viewpoint, of the tyrosine molecules which are present in the protein. The species specificity of serum and other proteins appears to be linked in some way with these groupings, although it must not be overlooked that antigenic specificity is not always due to aromatic groupings. The experiments of Landsteiner and Barron and those described here furnish some additional support for the view that the tyrosine molecules play an important part also in determining antigenicity, a view which still rests mainly on the experiments of Wells [1908] and later authors, who have shown that gelatin, a protein deficient in aromatic amino-acids, is non-antigenic.

Summary.

The action of acid and alkali on the power of horse-serum-proteins to react with antibodies to the untreated serum-proteins has been investigated with special reference to the precise influence of solutions of definite p_H values. The results agree with the earlier observations of other authors (Wells, *etc.*) that alkalis have a more marked destructive action than have acids. With moderate degrees of alkalinity, however (up to p_H 11 or so) there is very little loss of these antigenic properties in 24 hrs. at 19°.

In view of the findings of Landsteiner and Barron [1917] that the conversion of non-antigenic alkali-treated-proteins into xanthoproteins results in a regeneration of antigenic properties, attempts have been made to effect the same change by iodination. Some success was obtained, but iodination is not

as efficient as is nitration in regenerating antigenic properties in these alkali-treated proteins. The significance of these findings in relation to the chemistry of protein antigens is discussed.

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CXLIII. STUDIES ON THE RELATIONSHIP BETWEEN CHEMICAL CONSTITUTION AND PHYSIOLOGICAL ACTION.

IV. THE INHIBITORY ACTION OF CERTAIN SYNTHETIC URETHANES ON THE ACTIVITY OF ESTERASES.

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Part III [Stedman and Stedman, 1931] of this series of communications was concerned with an examination of the action towards liver esterase of the group of urethanes which had previously been shown to behave pharmacologically as "parasympathetic stimulants," and it was demonstrated that, while such urethanes possessed the power of inhibiting the action of this enzyme to a very high degree, other urethanes and bases did not produce a similar effect. It was therefore concluded that a relationship between constitution and inhibitory action towards liver esterase existed analogous to that between constitution and physiological action in the same group. This result afforded exceedingly strong, if not conclusive, evidence in favour of Loewi and Navratil's [1926] views regarding the mechanism of the action of physostigmine on the heart, and further appeared to indicate the nature of the enzyme which, according to Engelhart and Loewi [1930] and to Matthes [1930], brings about the destruction of acetylcholine and of the vagus substance. It is clear from Loewi's work, however, that the liver is not normally directly responsible for the destruction of the vagus substance, which, in his original experiments, was shown to be caused by aqueous extracts of the frog's heart. It therefore appeared to be desirable to examine the inhibitory action of the above group of urethanes towards serum esterase, particularly as Engelhart and Loewi, Matthes, and Plattner have shown that both whole blood and serum are capable of destroying acetylcholine. At the same time the actions of the urethanes on two other enzymes, namely pancreatic lipase and phosphatase, the normal substrates of which are esters, have been examined in order to determine the extent to which the inhibitory action of the urethanes is specific.

Pancreatic lipase.

Enzyme preparation. The pancreatic lipase was prepared from pig's pancreas according to Willstätter and Waldschmidt-Leitz's method [1923]. The minced glands were desiccated by successive treatment with acetone and ether and the fibrous material was separated from the dry preparation by pounding it in a mortar and then shaking it through a fine sieve. The fine powder so obtained was extracted for 4 hours at 30° with 87 % glycerol (32 cc. for 2 g. of powder), centrifuged, and the extract stored in this form. Immediately before use, 15 cc. of this extract were diluted to 60 cc. with water, and again centrifuged to remove a fine precipitate which separated.

Hydrolysis of olive oil. The influence of miotine hydrochloride on the hydrolysis of olive oil by pancreatic lipase was first examined. Into each of two small stoppered bottles 2·5 g. of olive oil were weighed and 2 cc. of ammonia-ammonium chloride buffer (p_H 8·9) were then added. The contents of one bottle were now treated with 11 cc. of the above enzyme extract and those of the other with 11 cc. of the same extract containing 5 mg. of miotine hydrochloride which had been dissolved in it one hour previously. Each bottle was shaken for 3 minutes to emulsify the oil and then placed in a thermostat at 30° for one hour. The contents of each bottle were now washed with 100 cc. of rectified spirit into a flask, 20 cc. of ether added to dissolve the oil and the solutions titrated with 0·727*N* alcoholic potassium hydroxide, using thymolphthalein as indicator. In each case 6·8 cc. of the alkali were used. A control, using 11 cc. of water in place of the enzyme solution, required 2·4 cc. of the alkali. It is clear that miotine exerted no inhibitory action.

Hydrolysis of tributyrin. In order to follow the hydrolysis of tributyrin, the stalagmometric method described in Part III was employed. The above diluted extract (1 cc.) of pancreatic lipase was mixed with 2 cc. of phosphate buffer (p_H 7·9) and diluted to 10 cc. with water. One cc. of this preparation was treated with 12 mg. of miotine hydrochloride dissolved in 1 cc. of water and allowed to stand for one hour, when 1 cc. of the mixture was used in a hydrolysis experiment. For the control, 1 cc. of water was employed in place of the solution of miotine hydrochloride. The following figures represent the diminution in the drop number in successive periods of 20 minutes: control, 15, 23; in the presence of miotine, 14, 23. Miotine is thus without inhibitory action on the hydrolysis of tributyrin by pancreatic lipase.

Hydrolysis of methyl butyrate. The method employed was at first identical with that used in Part III with liver esterase, except that the reaction mixture contained a high percentage of glycerol which activates as well as stabilises the lipase. Into a 100 cc. graduated flask were introduced 50 cc. of a 50 % solution of glycerol and 20 cc. of buffer (1 part 2·5 *N* NH_3: 2 parts 2·5 *N* NH_4Cl; p_H 8·9) and the mixture was then warmed to 30° in a thermostat. One cc. of methyl butyrate was dissolved as completely as possible in this by shaking, when 20 cc. of the diluted extract of pancreatic lipase were added and the volume was

made up to 100 cc. with water. The flask was again placed in the thermostat, 20 cc. of the mixture being immediately withdrawn, run into a mixture of 25 cc. of 0·2 *N* hydrochloric acid with 20 cc. of water and titrated with 0·2 *N* sodium hydroxide, using bromocresol purple as indicator. Similar volumes of the mixture were withdrawn at intervals of 20 minutes and titrated in the same way. In the experiment designed to test the inhibitory action of miotine, 12 mg. of the hydrochloride were dissolved in 25 cc. of the diluted extract of the enzyme and allowed to stand for one hour; 20 cc. of this solution were then used in a hydrolysis experiment. The following results were obtained, the figures representing the number of cc. of 0·2 *N* alkali required to titrate the acid liberated in 20 cc. of the reaction mixture in 20, 40 and 60 minutes respectively: control, 1·75, 2·7, 3·4; in the presence of miotine, 1·15, 1·85, 2·35. A small inhibitory effect is apparent, although it is much smaller in magnitude than with liver esterase.

Willstätter and Memmen [1924] have shown that calcium oleate exerts a marked activating action on the hydrolysis of methyl butyrate by pancreatic lipase. Another experiment was therefore carried out in the presence of this activator. The procedure was identical with that outlined above except that 2 cc. each of 2 % sodium oleate and calcium chloride solutions were added to the reaction mixture after the addition of the enzyme but before making up to volume. In view of the activation caused by this addition, only 5 cc. of the diluted glycerol extract of lipase were used. Nevertheless the same amount of miotine was employed. The following figures are typical of the results obtained: control, 2·25, 4·1, 5·05; in the presence of miotine, 1·7, 3·15, 4·25. Owing to the fact that the hydrolysis of methyl butyrate by pancreatic lipase does not take a linear course, possibly because of the changes which occur in the p_H of the solution, it is not possible to calculate in a simple manner the percentage inhibition produced by the miotine. Nevertheless it is clear from the figures that the inhibition caused by the miotine in this experiment is of the same order of magnitude as that produced by the same quantity in the absence of calcium oleate, although only one fifth of the amount of enzyme was required in the latter experiment.

An experiment similar to that last described was also carried out with the hydrochloride of the methylurethane of *m*-dimethylaminophenol, this particular urethane being chosen because it had proved to be the most active of the urethanes examined in inhibiting the hydrolysis of methyl butyrate by liver esterase. About 10 mg. of the urethane were employed, the remaining details being identical with those described above. The results are shown by the following figures: control, 2·05, 3·9, 4·75; in the presence of the urethane, 1·45, 2·9, 3·85. A small inhibitory effect is again apparent.

In view of the possibility that the glycerol, necessarily present in the above solutions, might diminish the inhibitory action of the urethanes, the influence of glycerol on the inhibitory action of miotine towards liver esterase was examined. An acidified and dialysed extract of liver powder, similar to those

described in Part III, was employed, the hydrolysis of methyl butyrate being followed under the conditions described in that paper with the modification that 50 cc. of water were replaced by 50 cc. of 50 % glycerol. As inhibitor, 0·01 mg. of miotine hydrochloride was employed. The following titration figures were obtained: control (without glycerol), 1·85, 3·8, 5·5; control (with glycerol), 1·85, 3·65, 5·45; in the presence of miotine (without glycerol), 0·55, 1·5, 2·2; in the presence of miotine and glycerol, 0·55, 1·4, 2·0. It is evident that the glycerol was without influence either on the activity of the esterase or on the inhibitory activity of miotine. The experiment further serves to illustrate the much greater sensitivity of liver esterase to miotine. With only a thousandth part of the amount used with pancreatic lipase a greater inhibition was produced.

Kidney phosphatase.

The preparation of kidney phosphatase employed was obtained by Erdtman's method [1927]. 600 g. of pig's kidneys were minced, suspended in 500 cc. of water to which much toluene had been added, and incubated for 2 days at 37°. The mixture was then filtered through a fine metal strainer, and the turbid filtrate treated with 1½ litres of rectified spirit. The precipitate so produced was filtered, stirred with 500 cc. of alcohol and again filtered, this process being repeated twice. It was then similarly treated with 500 cc. of ether, dried in the air and ground in a mortar. Extracts of the phosphatase were prepared by shaking 4 g. of this dry powder with 80 cc. of $N/40$ ammonia for 1½ hours and removing the solid material in the centrifuge.

The substrate employed was sodium glycerophosphate. To a mixture of 10 cc. of 5 % sodium glycerophosphate, 10 cc. of buffer (ammonia-ammonium chloride, p_H 8·9) and 60 cc. of water, previously warmed to 30° in a thermostat, were added 10 cc. of the above solution of phosphatase. The total volume was then made up to 100 cc. with water and the solution replaced in the thermostat. 20 cc. of the mixture were immediately withdrawn and run into 10 cc. of 10 % trichloroacetic acid. The free phosphate was then estimated in 25 cc. of the filtrate by precipitating it as phosphomolybdic acid, dissolving the latter after filtration in 10 cc. of 0·21N NaOH and titrating the excess alkali with 0·0995N nitric acid. Similar withdrawals and estimations were made at fixed intervals. A typical experiment will suffice to illustrate that miotine is without action on kidney phosphatase. 12 mg. of miotine hydrochloride were dissolved in 12 cc. of the enzyme solution and, after an interval of one hour, the phosphatase activity of 10 cc. of the solution was determined, a control being carried out simultaneously. The following figures represent the cc. of nitric acid required to neutralise the excess alkali in estimations on samples withdrawn immediately and after 80 minutes respectively: control, 18·2, 8·9; with miotine, 18·6, 8·9.

Serum esterase.

In order to examine the inhibitory action of the various urethanes towards serum esterase, the serum of the guinea-pig was chosen, since the blood of this

animal is known to contain a relatively high amount of the enzyme. 2–3 cc. of the serum, the exact volume depending upon the esterase activity of the sample employed, were treated with 2 cc. of phosphate buffer of p_H 7·9 and the mixture was diluted to 10 cc. with water. One cc. of this solution was mixed with 1 cc. of a solution of the urethane under examination and allowed to stand for about an hour, when the esterase activity of 1 cc. of the mixture was determined by the stalagmometric method described in Part III. In the control experiment, the solution of the urethane was replaced by an equal

Table I. *Inhibition of serum esterase by various urethanes.*

Substrate: tributyrin. T = 20°. p_H = 7·9.

Inhibitor*	Final conc. of inhibitor ($M \times 10^{-3}$)	Decrease in drop number in 20 mins.	Decrease in drop number in 40 mins.	Percentage inhibition
Phenyl series:				
Control	—	11	21	—
m-HCl	4	5	11	48
p-HCl	4	8	16	24
o-HCl	4	8	15	29
Control	—	13	25	—
m-MeI	400	5	9	64
p-MeI	400	1	4	84
o-MeI	400	1	3	88
Miotine series:				
Control	—	11	23	—
m-HCl	400	1	2	91
,,	40	5	10	57
p-HCl	400	2	4	83
,,	40	4	10	57
o-HCl	400	1	3	87
,,	40	9	19	17
Control	—	14	27	—
m-MeI	4000	2	3	89
,,	400	11	22	19
p-MeI	4000	1	3	89
,,	400	13	23	15
o-MeI	4000	1	2	93
,,	400	9	17	37
Benzyl series:				
Control	—	12	23	—
m-HCl	40	2	5	78
,,	4	8	16	30
p-HCl	40	3	7	70
,,	4	9	18	22
o-HCl	40	6	12	48
Control	—	14	26	—
m-MeI	400	8	17	35
p-MeI	400	6	14	46
o-MeI	400	7	15	42

* *Phenyl series*; methylurethanes of the isomeric dimethylaminophenols.
Miotine series; methylurethanes of the isomeric α-hydroxyphenylethyldimethylamines.
Benzyl series; methylurethanes of the isomeric hydroxybenzyldimethylamines.

volume of water. The results obtained are given in Table I. The urethanes employed were identical with those used in Part III and are indicated by the same abbreviated names.

Liver esterase.

In view of the fact that serum from the guinea-pig was used in the above experiments it was thought that it would be of interest to examine the inhibitory actions of the various urethanes on the liver esterase from the same species. A preparation of this esterase was therefore made from a number of guinea-pig's livers using the same procedure as was employed in Part III in connection with liver esterase from the pig. The activity of the preparation towards methyl butyrate, however, proved to be much smaller than that from the pig and it was not, therefore, possible to follow the hydrolysis of simple esters by the technique employed in Part III. Despite its smaller activity towards methyl butyrate, this preparation nevertheless proved to be at least as active towards tributyrin as was the esterase from pig's liver. The experiments recorded in Table II were therefore carried out, using this substance as

Table II. *Inhibition of guinea-pig's liver esterase by various urethanes.*

Substrate: tributyrin. T = 20°. p_H = 8·0.

Inhibitor	Final conc. of inhibitor ($M \times 10^{-3}$)	Decrease in drop number in 20 mins.	Decrease in drop number in 40 mins.	Percentage inhibition
Phenyl series:				
Control	—	14	26	—
m-HCl	40	0	0	100
p-HCl	40	9	18	31
o-HCl	40	7	16	38
Control	—	14	27	—
m-MeI	400	6	13	52
p-MeI	400	3	8	70
o-MeI	400	7	16	41
Miotine series:				
Control	—	11	22	—
m-HCl	40	4	8	64
p-HCl	40	6	14	36
o-HCl	40	4	8	64
Control	—	13	24·5	—
m-MeI	400	5	10·5	57
p-MeI	400	5	11	55
o-MeI	400	5	10·5	57
Benzyl series:				
Control	—	13	24	—
m-HCl	40	3	8	67
p-HCl	40	7	16	33
o-HCl	40	4	9	63
Control	—	14	27	—
m-MeI	400	4	9	67
p-MeI	400	5	12	56
o-MeI	400	6	13	52

substrate. The preparations of the esterase employed were obtained by extracting the desiccated liver powder with dilute ammonia, acidifying this extract with acid and, after removing the precipitate thus produced, dialysing for about 3 days in collodion membranes. In the various experiments recorded,

1·5–3 cc. of such an extract were mixed with 2 cc. of phosphate buffer of p_H 8·0 and the mixture was diluted to 10 cc. with water. One cc. of this solution was mixed with 1 cc. of water or of a solution of the urethane under examination, allowed to stand for about an hour, and 1 cc. of the mixture used in a hydrolysis experiment. The results obtained with the various urethanes are recorded in Table II.

Summary and discussion.

In so far as they show that the hydrolysis of tributyrin by the esterase from the liver of the guinea-pig is inhibited by small concentrations of the urethanes which have been shown to behave as parasympathetic stimulants, the above experiments constitute an extension to the liver esterase from a second species of the results obtained with the pig and reported in Part III of this series. While we have not carried out any extensive experiments with liver esterases from other species, we have nevertheless submitted those from a few to a preliminary examination and have found in each case that their activity is inhibited by miotine when present in concentrations of the same order of magnitude as employed in the above experiments. It would therefore appear legitimate to conclude that the urethanes of the type under consideration inhibit the activity of liver esterases in general and irrespective of the species from which they are derived.

Our experiments further demonstrate that the inhibitory action of the urethanes is not restricted to the liver enzyme but extends to the esterase which is present in the blood-serum of the guinea-pig and of certain other species. In view of the fact that it has been shown by the authors mentioned in the introduction that both whole blood and serum are capable of destroying small amounts of acetylcholine, a process which is inhibited by physostigmine, this result was, perhaps, to be expected. Nevertheless, it must be emphasised that the experiments here recorded have been carried out using tributyrin as substrate. While it would seem probable that the same enzyme is concerned in both processes, this cannot at present be regarded as definitely established. There exists, indeed, some evidence to the contrary. Thus, Takahashi [1930], has recently compared the lipolytic activities of the sera from a number of species, using tributyrin as substrate, and has found that they fall in the following order: rabbit > cat > horse > man > dog > cattle. This order is in general agreement with the results of earlier workers and also with some unpublished experiments which we have carried out. On the other hand, the order in which the defibrinated blood from a number of species destroys acetylcholine is, according to Galehr and Plattner [1927], man > pig $\geqq$ cattle > dog > horse > rabbit > cat. The latter series is practically the reverse of that which holds for the hydrolysis of tributyrin and hence suggests either that different enzymes are concerned in the two processes or that some other factor, hitherto unrecognised, is involved. It is unlikely that a solution of this aspect of the problem will be possible until the blood-enzymes have been purified and

concentrated. Unfortunately such purification is attended with considerable difficulty on account of the quantity and ready solubility of the serum proteins. We are nevertheless at present engaged in an attempt to effect this purification.

Although the possibility thus exists that the hydrolysis of tributyrin and the destruction of acetylcholine are brought about by different serum enzymes, our results appear quite definitely to indicate that the enzymes, if specifically different, are of the same general nature. We have now established that the activities of both liver and serum esterases are inhibited by small concentrations of urethanes of the miotine type. Nevertheless, the above results show that the activity of pancreatic lipase, an enzyme which, although it resembles liver esterase in hydrolysing simple esters, differs from the latter enzyme in attacking fats relatively more rapidly, is not inhibited by miotine when its substrate is either a fat, in the form of olive oil, or tributyrin. When, however, its substrate is methyl butyrate, a simple ester, its action is inhibited by miotine, although a concentration of the drug which is high compared with that required with serum or liver esterase is necessary. Similarly, the hydrolysis of glycerophosphoric acid by kidney phosphatase, a process involving an ester of a different type from that attacked by liver esterase, is unaffected by miotine. It thus appears that the inhibitory action of the urethanes with which we are concerned is directed mainly towards true esterases, and it is therefore probable that the destruction of acetylcholine is brought about by an enzyme of the same nature.

Regarding the nature of the inhibitory action of the drugs in question, there appears little to be added to our comments in Part III. It was pointed out in that communication that the urethanes are esters and that combination between them and the enzyme probably involves the same mechanism as with the normal substrate, in which case the resulting inhibition would be due to the inability of the enzyme to hydrolyse the urethane. Some chemical evidence which supports this view may be mentioned. In Part I [1926] of this series it was mentioned that when aqueous solutions of certain of the urethanes are boiled, decomposition occurs with the production of methyl *iso*cyanate. This decomposition was erroneously referred to as hydrolysis, but it is clear, as pointed out by Aeschlimann and Reinert [1931] in a paper concerned with the pharmacology of urethanes of the same type, that no hydrolytic process is involved. This can be seen from the following equation:

$$N(CH_3)_2.CH(CH_3).C_6H_4.O.CO.NHCH_3 = N(CH_3)_2.CH(CH_3).C_6H_4.OH + CH_3CNO.$$

If, then, despite their structures as esters, these urethanes are not normally hydrolysed by ordinary chemical reagents but undergo a decomposition of a different nature, it is not difficult to understand the inability of esterases to bring about their destruction. The inhibitory action would be a direct consequence of this, provided the urethanes were endowed with high affinities for the enzyme.

The general relations between the nature of the urethane and its inhibitory activity towards liver esterase observed in Part III and discussed in that paper have again been encountered in the experiments described above and require no further comment. The order of activity of isomeric urethanes appears, however, to differ according to the source of the enzyme. While it would be in accordance with other observations that the enzymes from different species should exhibit differences in this respect, it is more difficult to understand why the esterases from the liver and serum of the same species should show similar differences. The obvious explanation, that the two latter enzymes are different substances, may be correct. We feel, however, that it would be wiser at the present time not to lay too much stress upon these small differences, which might conceivably be caused by impurities which are present in the enzyme solutions.

The authors desire to thank Dr C. P. Beattie of the Bacteriology Department of this University for providing them with the guinea-pig's serum and livers used in this work. The expense of this investigation has been largely defrayed by grants from the Earl of Moray Research Fund of this University.

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CXLIV. NOTE ON THE INTERNATIONAL STANDARD FOR THE ANTINEURITIC VITAMIN B_1.

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The vitamin B_1 preparation adopted as International Standard by the League of Nations Conference on Vitamin Standards, held in London in June 1931, is an adsorbate on fuller's earth obtained by Seidell's method [1916] from an extract of rice polishings, the extract being made with water containing 20 % alcohol and acidified with sulphuric acid to p_H 4·5 [League of Nations Health Organisation, 1931]. 3 kg. of the fuller's earth represent the antineuritic vitamin B_1 from 100 kg. of rice polishings. This adsorbate, which is prepared in the Medical Laboratory at Batavia (Java) and used in that district for the treatment of beriberi, formed the starting-point for the preparation of the crystalline vitamin B_1 product isolated by Jansen and Donath [1927].

Before its adoption as a vitamin B_1 standard, this "acid clay" adsorption product was investigated by several members of the Conference to ascertain its degree of suitability for use as a standard. At the request of the Accessory Food Factors Committee (of the Medical Research Council and Lister Institute) quantitative tests using different methods were made in various laboratories in this country and abroad; in these tests the adsorbate was used as a standard of comparison in the estimation of the potency of other vitamin B_1-rich substances—dried yeast, wheat embryo, *etc.* Standard specimens of these materials were distributed to the various laboratories and in the course of tests carried out at the Lister Institute the following results were obtained which seem worthy of record, as being possibly of general interest and of use to other workers in this field.

In the method of vitamin B_1 estimation employed in the following tests, the criterion adopted was the growth of young rats [Chick and Roscoe, 1929]. A basal diet was used (caseinogen 100; rice starch 300; salt mixture McCollum No. 185, 25; arachis oil 75; water 550; with cod-liver oil 50–100 mg. daily administered separately), to which the antidermatitis vitamin B_2 was added as a small daily ration of an autoclaved (4 hours, 120°, at p_H 5·0) watery yeast extract given in amount equivalent to 0·5 g. of the yeast (dry wt.) from which the extract was prepared [see Roscoe, 1930]. The standard degree of "normal" growth adopted was an average weekly increase in body weight of 10–14 g. While the tests were in progress there was an exceptionally cold period, lasting about two weeks, during which it was impossible to keep the animal room at the usual temperature and the growth of the rats under observation was checked. In calculating the average weekly increments in growth, those corresponding to this period have been omitted.

The vitamin B_1 standard adsorption product is a fine white powder easy to administer and readily taken. The minimal daily dose required to support an average weekly increase of 10–14 g. was found to be about 10 mg. (see Table I) and for convenience in preparing the doses the material was diluted ten-fold with pure dextrin and thus administered. The results of the series of tests summarised in Table I show the standard to be about 6 and 12 times as

Table I. *Comparative (antineuritic) vitamin B_1 value of* (a) *the standard adsorption product from rice polishings*, (b) *dried yeast*, (c) *wheat embryo, and* (d) *Peters's antineuritic concentrate from yeast.*

(Vitamin B_2 supplied as autoclaved yeast extract, daily dose≡0·5 g. yeast (dry wt.).)

Material	Daily dose g.	No. of rats used	Av. weekly increment in weight during 5 weeks' observation g.	Av. weekly increment in growth during 1st week g.	Av. weekly increment in growth during 5th week g.
(*a*) International Standard ...	0·025	1	16·2		
	0·012	6	14·3	15·0	13·5
	0·009	4	11·5	15·0	8·2
	0·006	4	7·7	8·5	6·5
International Standard after 10–12 months in refrigerator	0·010	2	11·4	19·0	9·0
	0·0075	2	9·7	13·0	4·0
International Standard after 10–12 months at 37°	0·010	2	10·6	14·5	11·0
	0·0075	2	8·1	14·0	5·0
(*b*) Dried yeast	0·10	3	17·5	17·5	12·0
	0·075	5	14·6	17·2	11·0
	0·05	5	10·9	9·7	11·5
(*c*) Wheat embryo ...	0·20	4	16·4	17·0	10·0
	0·15	4	15·2	17·7	9·2
	0·10	6	10·3	13·5	7·2
	cc.				
(*d*) Peters's antineuritic concentrate from yeast (0·1 cc.≡0·6 g. yeast dry weight)	0·10	4	17·2	18·0	17·0
	0·075	2	17·4	21·0	15·0
	0·05	3	15·4	17·7	11·0

potent respectively as the samples of dry yeast and wheat embryo tested. The International unit of vitamin B_1 is defined as the "antineuritic activity of 10 milligrams of the international standard adsorption product" [Report, 1931, p. 76]. The sample of dried yeast examined would therefore contain 16–18 International vitamin B_1 units per g. and the sample of wheat embryo 8 units per g.

It has frequently been found that when young rats are fed on a simplified "complete" diet containing the minimum amounts of B-vitamins for "normal" growth, the rate of increase in weight may show a falling off after a few weeks; in the estimation of vitamin B_1 this may happen towards the end of the five weeks' period of the test (see Table I, column 5). In the present experiment this occurred to some extent when vitamin B_1 was given as dried yeast, but to a greater extent when wheat embryo or the standard adsorption material from rice polishings was used and more especially after the latter material had been stored for a year (Table I).

It is noteworthy that in most investigations of the B-vitamins, those factors have been studied as they occur in yeast and yeast products. Failure in continued growth on a "complete" experimental diet has frequently been observed when the yeast products have been purified or when the constituents of the B-vitamin complex have been derived from other natural substances. To explain the relative failure when vitamin B_2 was supplied by a preparation from egg-white rather than by an autoclaved yeast extract Chick and Copping [1930] and Roscoe [1930] postulated the existence of factor Y, an unknown constituent of the yeast vitamin complex abundantly present in yeast and some other foods and lacking in egg-white. In the present tests, however, factor Y would be provided in the autoclaved yeast extract given as source of vitamin B_2. The relatively heat-labile vitamin B_4, found by Reader [1929, 1930, 1, 2] to be necessary for rat growth, would be supplied in the acid clay ration, seeing that the crystalline vitamin B_1 product finally obtained from this material by Jansen and Donath's method was found by Jansen *et al.* [1930] to be an unusually rich source of vitamin B_4.

It is probable that our knowledge of the B-vitamin complex might be extended and some of the discrepancies characteristic of the subject removed by a further study of these vitamins as they occur in natural materials other than yeast. In the meantime the existing evidence suggests the presence in yeast of other essential dietary factors in addition to those of the vitamin B complex which have been already recognised.

Stability of the standard vitamin B_1 adsorption product. This material was reported from the Medical Laboratory, Batavia, to possess great stability. In the present investigation tests made after storage for 10–12 months, both in a refrigerator and in an incubator room maintained at 37°, showed little, if any, deterioration to have taken place in the original vitamin B_1 potency (Table I).

Content of vitamin B_2. A few growth tests were made on young rats receiving diets in which the standard adsorption product was given as source of vitamin B_2, and vitamin B_1 was provided as Peters's purified concentrate from yeast (method of Chick and Roscoe [1928]). Only an insignificant degree of growth (3–4 g. weekly) was obtained with daily doses of 0·05 and 0·1 g.; with 0·25 g. daily the average weekly increment in weight was only 6·5 g. The vitamin B_1 content of the standard material is therefore more than 25 times, and probably at least 50 times, greater than its content of vitamin B_2 and the amount of the latter contained in the small daily dose of 10 mg. required to supply vitamin B_1 might be regarded as negligible.

This standard vitamin B_1 could therefore be used as a source of vitamin B_1, sufficiently free from vitamin B_2, in diets destined for work on the latter vitamin and would provide a much more convenient and economical material for the purpose than Peters's vitamin B_1 concentrate from yeast, which is only obtained after an elaborate and lengthy process of preparation.

SUMMARY.

1. The preparation adopted as International Standard for the antineuritic vitamin B_1 (adsorption product on fuller's earth from an acid, watery extract of rice polishings) has been found exceedingly potent (daily rat dose about 10 mg.), convenient to handle and easy to administer.

2. It possesses a satisfactory degree of stability when preserved for a year either at room temperature, at 37° or in a refrigerator.

3. Its content of vitamin B_2 was found to be negligible in comparison with its vitamin B_1 potency. This material should therefore serve as a useful source of vitamin B_1 in diets designed for the study of vitamin B_2.

In conclusion our best thanks are due to Prof. B. C. P. Jansen for the supply of the standard vitamin B_1 adsorption product with which the above tests were made.

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CXLV. EQUOL, A NEW INACTIVE PHENOL ISOLATED FROM THE KETOHYDROXY-OESTRIN FRACTION OF MARES' URINE.

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The discovery by Zondek [1930] that the urine of pregnant mares contained large amounts of the oestrus-producing hormone has resulted in the widespread adoption of this material as the starting-point for the large scale manufacture of the hormone. It appears that the isolation of the crystalline hormone from mares' urine in a chemically pure condition is a somewhat more difficult problem than its isolation from human urine. De Jongh, Kober and Laqueur [1931], Nieuwenkamp and Kober [1931], and Dingemanse *et al.* [1931], working in Laqueur's laboratory, have reported that the crystalline active substance present in mares' urine is identical with their crystalline "menformon" (ketohydroxyoestrin) isolated from human urine. These workers found that the crude crystallisates were contaminated with other substances possessing considerably lower melting-points. By frequent crystallisations they were able however to separate the pure hormone.

More recently Girard *et al.* [1932] have shown that the hydroxyketone fraction of mares' urine concentrates contains, in addition to ketohydroxyoestrin, two other active crystalline substances which appear to have one more double bond than the former and which differ in their optical rotations.

It is clear therefore that processes, which when applied to human urine extracts yield pure ketohydroxyoestrin, may fail to do so when applied to mares' urine owing to the presence of other crystalline substances having similar solubilities and similar chemical properties.

Recently the authors have had the opportunity of examining a large quantity of crude mares' urine extract which was put at their disposal by Schering-Kahlbaum A.G., Berlin.

The total phenolic fraction was separated by the method previously described [Marrian and Haslewood, 1932], and this was then fractionated between benzene and aqueous alcohol. The benzene-soluble fraction containing the ketohydroxyoestrin fraction [*cf.* Butenandt, 1930] was then distilled in a

high vacuum. This process, when previously applied to human urine extracts, invariably yielded a crystalline or semi-crystalline sublimate from which it was easy to isolate pure ketohydroxyoestrin by washing with cold ether and crystallising from aqueous alcohol. In the case of the mares' urine extract, the product was found to be a clear, pale yellow glass. This material was all easily soluble in cold ether and no crystalline substance with a melting-point within 10° of ketohydroxyoestrin could be separated by direct crystallisation. The ketohydroxyoestrin was therefore separated by solution in the minimum of dilute alkali and ether-extraction [*cf.* Veler, Thayer and Doisy, 1930].

From the aqueous alkaline solution, on saturation with carbon dioxide, large amounts of a buff-coloured amorphous solid were precipitated. This substance crystallised readily from benzene or aqueous alcohol in nearly colourless needles which melted after several recrystallisations at 189°–190·5°. On analysis it was found to have the formula $C_{15}H_{14}O_3$. It exhibited no oestrus-producing action when injected into ovariectomised mice in doses as large as 0·086 mg. (1 m.u. of trihydroxyoestrin = 0·000125 mg.).

On methylation with methyl sulphate and sodium hydroxide a compound was formed which analysis proved to be a dimethyl ether. It was clear therefore that two oxygen atoms were present in phenolic hydroxyl groups.

Acetylation and benzoylation yielded a diacetate and a dibenzoate respectively, which suggested that the third oxygen atom was not present in a non-acidic hydroxyl group.

Both the phenol itself and its dimethyl ether were methylated, acetylated and benzoylated under various conditions, but in no case was any indication obtained of a third reactive hydroxyl group. Since it was possible that a third hydroxyl group might be present which was resistant to esterification, an experiment was carried out in which the dimethyl ether was treated with Grignard's reagent (CH_3MgI) in amyl ether. No evolution of methane occurred. We are of the opinion that these experiments definitely exclude the possibility of a third hydroxyl group.

Neither the phenol itself nor its dimethyl ether could be induced to react with hydroxylamine or 2 : 4-dinitrophenylhydrazine which suggested that the third oxygen atom was not ketonic, although the possibility of a non-reactive ketonic group could not be excluded.

The evidence, although of a negative character, suggests that the third oxygen atom may be present in an ether linkage. Determination of the methoxyl content of the dimethyl ether, however, proved the absence of methoxyl groups or other ether groups in the phenol itself which would readily yield volatile iodides on hydrolysis with hydriodic acid. The authors are inclined to favour the view that the third oxygen atom is present as a cyclic ether.

In view of the source of this new phenolic compound, the name "equol" is suggested as being suitable.

Experimental.

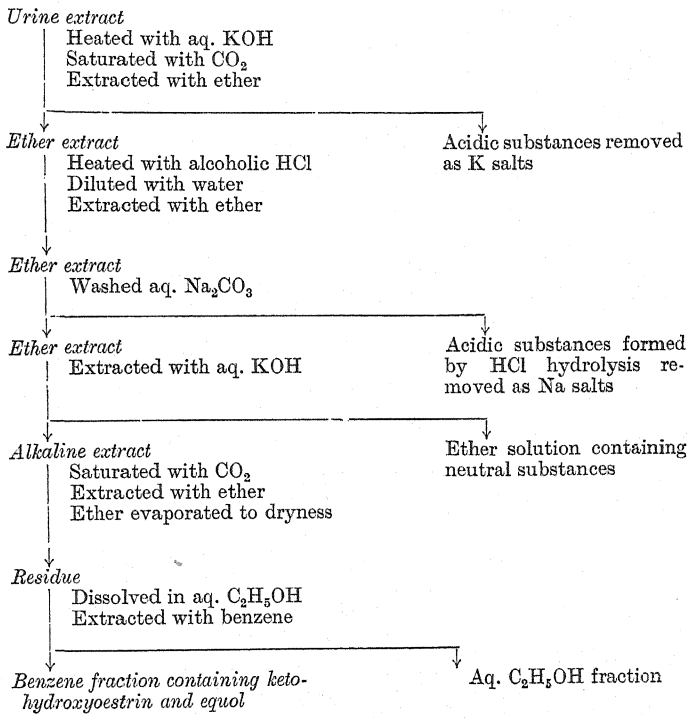
Methods. Melting-points were determined with short-stemmed Anschütz thermometers. No corrections were applied.

Optical rotations were observed in a 0·5 dm. Fischer micro-tube, using the mercury green line.

Micro-determinations of carbon, hydrogen, halogens and methoxyl groups were carried out by Dr Ing A. Schoeller, Berlin. Micro-molecular weight determinations were made by Rast's method. All substances before analysis were dried over phosphorus pentoxide in high vacuum.

Isolation of equol. The scheme adopted for the separation of the keto-hydroxyoestrin fraction of the mares' urine extract is shown in Table I.

Table I.



The benzene solution containing the ketohydroxyoestrin and equol was evaporated to dryness and distilled for 12 hours at 100–110°/0·01–0·03 mm. Hg. This distillate was discarded. The temperature was then raised to 160–180° for several days. The glass-like sublimate was collected, and dissolved in the minimum amount of hot dilute aqueous sodium hydroxide, diluted heavily with water and extracted repeatedly with ether to remove the ketohydroxy-oestrin. The aqueous alkaline fraction was then saturated with carbon dioxide and the crude equol, which was precipitated, filtered off, washed thoroughly

with water and dried. The yield from 1 kg. of crude urine extract, representing about 500 litres of urine, was about 3 g.

Purification and properties of equol. The crude equol after decolorising with charcoal in alcoholic solution was crystallised from aqueous alcohol and from benzene. A sample crystallised once from aqueous alcohol and four times from benzene melted at 189–190·5°.

Equol was readily soluble in ethyl and methyl alcohols, ether, ethyl acetate and acetone. In cold benzene, cold water, chloroform and light petroleum, the solubilities were low. In view of these solubilities, it is remarkable that equol appears in the benzene fraction rather than the aqueous alcoholic fraction in the fractionation of the total urine-phenols. The fact was confirmed however by a separate experiment on a pure sample of equol.

Equol gave strong Millon and xanthoproteic reactions. No colour was given with ferric chloride either in dilute aqueous solution or in aqueous alcoholic solution.

Optical rotation of equol. Rotations were determined in alcoholic solution on several different samples of equol of different degrees of purity. The results are shown in Table II.

Table II. *Optical rotation of equol.*

Times recryst.	M.P. ° C.	Wt. taken in mg.	Alcohol cc.	Temp.	α	$[\alpha]_{5461}$	Batch No.
2	184–187	3·15	0·40	20°	−0·086°	−21·8°	1
2	184–187	11·06	0·50	19°	−0·236°	−21·3°	1
2	184–187	3·20	0·54	20°	−0·056°	−18·8°	1
1	187–189	9·35	0·50	19°	−0·170°	−18·2°	2
1	187–189	9·92	0·50	19°	−0·226°	−22·8°	2
5	189–190·5	9·68	0·50	20·5°	−0·212°	−21·9°	2*a*
5	189–190·5	7·95	0·50	21°	−0·202°	−25·4°	2*a*
					Mean	−21·5°	

Although there is not perfect agreement between the figures obtained by different determinations on the same batch, the results are as satisfactory as can be expected working with such small amounts of material. It is clear that the rotation does not vary appreciably with the extent of purification and accordingly it is concluded that it is a definite property of equol itself rather than due to a small amount of optically active impurity.

Analysis of equol. Sample M.P. 189–190·5°.

4·470 mg. gave 12·145 mg. CO_2, 2·360 mg. H_2O;
4·310 mg. gave 11·695 mg. CO_2, 2·290 mg. H_2O;
0·23 mg. in 2·70 mg. camphor, Δ, 15·0°, 14·6°;
0·21 mg. in 2·04 mg. camphor, Δ, 16·0°, 16·0°.

(Found: C, 74·11, 74·02; H, 5·91, 5·94 %. Mol. wt., 230, 236, 255. $C_{15}H_{14}O_3$ requires: C, 74·38; H, 5·78 %. Mol. wt., 242.)

Equol dimethyl ether. 80 mg. of equol (M.P. 184–187°) were dissolved in about 10 cc. of $N/2$ sodium hydroxide and shaken for several hours with 2 cc. of dimethyl sulphate. The solid which separated was filtered off, washed

thoroughly with water, dried and crystallised from absolute ethyl alcohol. 45 mg. of the crystallised product, M.P. 88·5–89·5°, were obtained. The product was insoluble in aqueous alkali and gave no Millon reaction.

4·550 mg. gave 12·205 mg. CO_2, 2·630 mg. H_2O;
5·300 mg. gave 14·415 mg. CO_2, 3·130 mg. H_2O;
3·802 mg. gave 6·455 mg. AgI (micro-Zeisel).
0·24 mg. in 2·58 mg. camphor, Δ, 14·5°, 15·0°;
0·22 mg. in 3·32 mg. camphor, Δ, 9·5°.

(Found: C, 75·86, 75·56; H, 6·70, 6·73 %; CH_3O, 22·59 %. Mol. wt., 251, 245, 244. $C_{15}H_{12}O.(OCH_3)_2$ requires: C, 75·55; H, 6·66; CH_3O, 22·96 %. Mol. wt., 270.)

Equol diacetate. 53·9 mg. of equol (M.P. 184–187°) were heated with 3 cc. acetic anhydride for 3 hours at 100°. After cooling, excess of water was added and the precipitated acetate washed and dried. The crude product was recrystallised from absolute methyl alcohol. 25 mg. of the recrystallised acetate, M.P. 122·5°, were obtained.

4·281 mg. gave 10·900 mg. CO_2, 2·130 mg. H_2O;
0·23 mg. in 1·82 mg. camphor, 1st M.P. Δ, 20°.

(Found: C, 70·08; H, 5·58 %. Mol. wt., 316. $C_{15}H_{12}O.(OOC.CH_3)_2$ requires: C, 69·94; H, 5·52 %. Mol. wt., 326.)

Equol dibenzoate. 63·8 mg. of equol (M.P. 164–167°) were heated for a few minutes with 3 cc. benzoyl chloride. The mixture was cooled and then shaken for several hours with an excess of 2*N* NaOH. The crude benzoate which separated was filtered off, washed thoroughly and dried. The product was recrystallised twice from a mixture of equal parts of chloroform and absolute methyl alcohol. About 80 mg. of recrystallised benzoate were obtained. The dibenzoate showed a clear double melting-point. At 187–189° it melted to a translucent liquid; at 223·5–225°, the melt cleared sharply. Just below 223° a vivid play of colours was observed in the melt. Clear evidence of liquid crystal formation was gained by examination of the melt by polarised light.

4·719 mg. gave 13·080 mg. CO_2, 2·080 mg. H_2O;
0·29 mg. in 2·62 mg. camphor, 1st M.P., Δ, 10·0°;
0·186 mg. in 1·68 mg. camphor, 1st M.P., Δ, 10·5°.

(Found: C, 76·83; H, 4·98 %. Mol. wt., 445, 438. $C_{15}H_{12}O.(OOC.C_6H_5)_2$ requires: C, 77·33; H, 4·89 %. Mol. wt., 450.)

Summary.

A dihydroxyphenol, $C_{15}H_{12}O(OH)_2$, M.P. 189–190·5°, $[\alpha]_{5461} = -21·5°$, has been isolated from the ketohydroxyoestrin fraction of pregnant mares' urine. This substance has been designated equol.

Equol has no oestrus-producing action.

The following derivatives of equol have been prepared: dimethyl ether, $C_{15}H_{12}O(OCH_3)_2$, M.P. 89°; diacetate, $C_{15}H_{12}O(OOCCH_3)_2$, M.P. 122·5°; dibenzoate, $C_{15}H_{12}O(OOCC_6H_5)_2$, M.P. 187–189° and 223·5–225° (liquid crystal formation).

The authors wish to express their sincere thanks to Prof. W. Schoeller of Schering-Kahlbaum A.G., Berlin, for supplying the extract used in this work and for giving them information concerning its preparation.

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CXLVI. SURFACE FILMS OF OESTRIN DERIVATIVES.

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Introduction.

This paper describes the results of surface pressure and surface potential measurements on various derivatives of oestrin, spread in the form of unimolecular films on water surfaces. The work was undertaken in the hope of throwing light on the constitution of the molecules, as well as for the intrinsic interest attaching to the surface properties of substances of such biochemical importance as the oestrous hormone.

The recent work of Butenandt, Marrian, Doisy, and their collaborators [*cf.* Marrian, 1932] has shown that at least two oestrus-producing substances are present in the urine of pregnant women, namely (*a*) trihydroxyoestrin $C_{18}H_{21}(OH)_3$ and (*b*) ketohydroxyoestrin $C_{17}H_{21}CO(OH)$. The former compound contains two hydroxyl groups one of which is phenolic; the latter one phenolic hydroxyl and one ketonic group. The fact that trihydroxyoestrin may be converted into ketohydroxyoestrin by distillation with sodium bisulphate clearly shows the close chemical relation between the two compounds and suggests that the two alcoholic hydroxyl groups in the former compound, from which water is eliminated to form the hydroxyketone, are attached to adjacent carbon atoms.

Little so far is known with certainty concerning the constitution of these compounds. It is evident that at least one benzene nucleus is present, while the work of Doisy and his colleagues [Thayer, Levin and Doisy, 1931, 1, 2] suggests the presence of one double bond which reacts readily with halogens. Butenandt and co-workers [1932] have recently discussed the question of the presence or absence of a reactive double bond. They suggest tentatively that an "enolic" double bond is present in the molecule in addition to the three double linkages in the benzene ring.

If it is assumed that a double bond is present, then it is probable that the carbon skeleton is a substituted partially reduced three benzene ring system.

Such a compound might belong to the group of (*a*) diphenylbenzene, (*b*) phenanthrene or anthracene, (*c*) phenylnaphthalene. If the double bond were not present these structures would still be possible if a bridged ring were present.

With the assumption that no double bond or bridged ring is present a condensed four benzene ring structure seems most probable. Another possibility is a structure based on the Windaus-Wieland formula for sterols. The well known facts that, during pregnancy, there is some disturbance of sterol metabolism, and that the sterol-like compound, pregnandiol, is present together with the hormone in the urine of pregnant women, might suggest a connection between oestrin and the sterols. On the basis of Rosenheim and King's [1932] recently suggested chrysene structure for sterols, the condensed four ring structures are again supported. In this connection, however, if the chrysene suggestion proves to be incorrect, other four ringed structures containing one or more five membered rings are possible. Certain of these skeletons appear less probable on consideration of the chemical evidence. The diphenylbenzenes and phenylnaphthalenes ought easily to yield aromatic acids of lower molecular weight on fusion with potash, but in numerous experiments by two of us (G. A. D. H. and G. F. M.) no indication of such products has been found in brief fusions. The main product of potash fusion has been found to be (unpublished experiments by the above authors) an eighteen carbon atom dicarboxylic acid. Since this is extremely stable to potash fusion it is more probable that a condensed ring structure is present. It will be seen that the surface film results are in accordance with this view, as also are the crystallographic measurements of which Bernal [1932] has given a preliminary account.

Butenandt and his collaborators [1932] have come to the conclusion that a phenanthrene structure is probable. While the findings in this paper provide support for this view, the possibility of four ring structures is equally probable.

Experimental.

The surface pressure (the outward force on a barrier in the surface dividing the film-covered surface from a clean water surface) was measured by the apparatus of Adam and Jessop [1926, 1] and Adam [1930, p. 37]. The substances were weighed out in small stoppered bottles, in quantities of 1–2 mg.; sufficient alcohol was added to dissolve the substance, and the solution was diluted to about 2 cc. with benzene. This solution was dropped on to the surface from a calibrated dropping pipette, employing the corrections for evaporation of the solvent described by Adam and Jessop [1926, 2]. The error in the measurement of the amount put on the surface is estimated as of the order of 3 %.

The films were examined for collapse by the method of Zocher and Stiebel [1930], in which a cardioid condenser is inserted through the bottom of the trough, giving intense dark-ground illumination of the surface. Collapsed aggregates show up in this way as bright spots on a dark ground, whereas perfect unimolecular films show nothing.

The change in the electrical potential at the air-liquid surface was measured by a modification of the method recently published by Schulman and Rideal [1931] an account of which will be published shortly. We are greatly indebted to Mr J. B. Harding for assistance in making the surface potential measurements.

Experimental results.

Trihydroxyoestrin, and ketohydroxyoestrin, also the acetyl derivative and the oxime of the latter, dissolved too rapidly in the water for any measurements to be made on the films. The methyl ether of the ketone and the trimethyl ether of trihydroxyoestrin formed unstable, rapidly collapsing films, on which no measurements could be made. The triacetyl derivative and the diacetyl derivative of the methyl ether of the trihydroxyoestrin, in which the two alcoholic hydroxyls are acetylated and the phenolic hydroxyl is methylated, were, however, found to give films stable up to about 7 dynes per cm., and collapse was slow even at higher pressures, so that fairly accurate measurements could be taken above 10 dynes. The results are shown in Figs. 1 and 2.

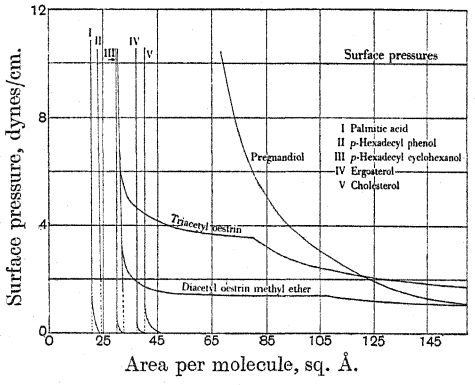


Fig. 1.

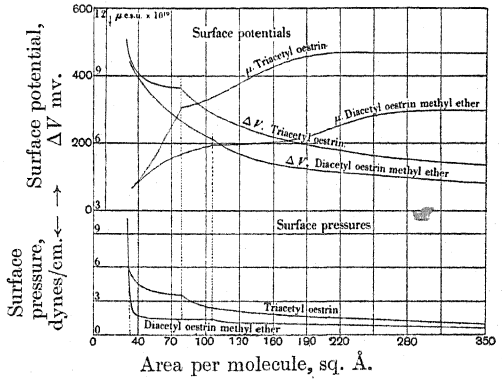


Fig. 2.

Both substances give, at large areas, gaseous films with only moderately large corrections to the perfect gas laws. On compression to about 100 sq. Å., per molecule, a sharp break occurs in the surface pressure area curves, which become nearly, but not quite, horizontal; there is only a slight increase in pressure down to 35 sq. Å. These breaks occur at 81 sq. Å. with the triacetyl compound, and at 109 sq. Å. with the diacetyl methyl ether. The surface pressures at the beginning of this transition region are about 3·6 and 1·5 dynes per cm. respectively; the curves were taken on distilled water and on $N/100$ potassium chloride solution. At the left-hand end of the transition region, the pressure rises sharply, giving a typical curve of a condensed film of the "close-packed head" type, which tends to an area 32·5 sq. Å. at no compression, with a probable error of about 1 sq. Å., for both substances.

These curves resemble, at first sight, the curves for aliphatic substances which give both gaseous and condensed films, but there is a very important difference; in the present case temperature has no detectable influence on the surface pressure at which the transition from gaseous to condensed film takes place, whereas with long-chain substances the surface pressure in the transition region rises steadily with temperature. The change in surface pressure with temperature is less than 0·05 dyne, per 10°, from 0° to 45°.

Fig. 2 shows the changes in surface potential ΔV, due to the film, and also the values of μ, calculated from Helmholtz's equation

$$\Delta V = 4\pi n\mu,$$

n being the number of molecules per sq. cm., and μ the average vertical component of the change in dipole moment of the part of the surface occupied by a molecule of the film, including the dipole moment of the film molecules themselves, and all effects due to redistribution of water molecules and of ions in the underlying solution. The breaks at the points where the gaseous films begin to change into the condensed films are clearly marked on the surface potential curves, at exactly the same areas as on the surface pressure curves. At large areas, although naturally ΔV decreases as the number of molecules in the film becomes smaller, μ increases and becomes practically constant, at 9×10^{-19} e.s.u. per molecule for the triacetyl compound, and $7{\cdot}5 \times 10^{-19}$ e.s.u. for the diacetyl methyl ether. There is a slight decrease in the gaseous film, as the area is reduced, before the discontinuity is reached; at smaller areas the values of μ decrease more rapidly, converging in the case of both compounds to a value about 4×10^{-19} e.s.u. for the condensed film. The surfaces were carefully searched at all areas to see if the surface consisted of two surface phases, the presence of which would be indicated by large fluctuations in ΔV. No fluctuations greater than the experimental error were found, except in the condensed films at pressures where the films were obviously collapsing.

Ultramicroscopic examination disclosed no sign of collapse of the films above about 35 sq. Å.; at smaller areas typical collapse aggregates were seen, forming slowly unless the pressure was above 7 dynes per cm.

The remaining curves in Fig. 1 are inserted for comparison, and include the surface pressure area curves for long-chain phenols [Adam, 1923], cholesterol and ergosterol [Adam and Danielli, unpublished], long-chain *cyclo*hexanols obtained by reduction of the phenols, and the dihydroxylic sterol pregnandiol recently isolated by Marrian [1929] and identified by Butenandt [1931], and for fatty acids on distilled water and other compounds giving the typical curve of closely packed long chains.

The *p*-dodecyl- and hexadecyl-*cyclo*hexanols were specially prepared for this work, in order to obtain an idea of the size of the *cyclo*hexane ring, standing upright and closely packed in the surface. The unreduced benzene ring had earlier been shown to pack to an area of 24 sq. Å. [Adam, 1923]; it was probable that the fully reduced *cyclo*hexane ring would occupy a good deal more space,

partly because of the bulk of the additional hydrogen atoms, and partly because it is a "puckered," not a plane ring. Both the dodecyl- and hexadecyl-*cyclo*hexanols formed condensed films of the "close-packed heads" type, having an area of about 30 sq. Å. at no compression, and being only slightly compressible. The cross section of the oestrin molecule, as packed in the condensed films, is thus about 8 % larger than that of the *cyclo*hexane group. As the temperature was raised, dodecyl*cyclo*hexanol appeared to pass into a liquid expanded film, having an area only slightly greater than that of the condensed film, about 35 sq. Å. at no compression. The investigation of this change was difficult, on account of the unusually small difference in area between the condensed and expanded films; the half expansion temperature under 1·5 dynes compression was $28° \pm 5°$. At high temperatures, the film of hexadecyl*cyclo*hexanol collapsed too rapidly for any expansion to be observed.

Pregnandiol gives a gaseous film at all pressures; the oestrin films resemble those of the *cyclo*hexanol derivatives much more closely than any of the others.

Discussion.

There can be little doubt that the molecules are lying flat, and moving about in the surface more or less independently in the gaseous regions of the films, *i.e.* at areas over 110 sq. Å. per molecule. Previous work has indicated that the molecules are always lying flat in the gaseous state of the films at large areas, though some degree of tilt may be caused by lateral compression. It is equally clear that the molecules are standing on end, closely packed, in the condensed regions of the curves, and therefore the cross-sectional area of the oestrin molecules, in their closest packing, is about 32·5 sq. Å., decidedly less than that of the sterols, and slightly greater than that of *cyclo*hexanol.

It is also certain that the vertical component of the electric moment per molecule is greater when the molecules are lying flat than when they are upright. This must indicate that the direction of the resultant dipole of the various polar groups in the molecule is inclined at a sharp angle to the plane of the molecule as a whole. In all probability this means that the two hydroxyl groups which have an alcoholic character are side by side, on the same side of the ring —*i.e.* they are in *cis*, not *trans*, relation to each other. If they were on opposite sides of the ring, they would neutralise one another. Further, since the dipole component perpendicular to the surface decreases very greatly as the molecules stand upright, it appears that these two hydroxyl groups are arranged with the bond between hydrogen and oxygen, or between oxygen and carbon, at a sharp angle to the plane of the ring.

The transition regions between the condensed and fully gaseous regions of the films indicate varying degrees of tilt in the molecules as the lateral compression is increased. The fact that the vertical dipole component decreases appreciably at areas greater than those corresponding with the discontinuities at 81 and 109 sq. Å., indicates that there is some tilting of the molecules away from the flat position before these areas are reached. There is, however, at the

areas where the discontinuities occur, a sudden decrease in the stability of the molecules lying flat; a comparatively slight increase in lateral compression has a much greater tilting effect here than at greater areas.

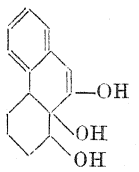
In interpreting the results of surface potential measurements, it must be remembered that μ is not simply the vertical component of the dipole moment of the film molecules, but includes terms of unknown amounts due to re-distribution of ions and re-orientation of water molecules, as Schulman and Rideal [1931] have pointed out. We are not in entire agreement with these authors as to the method of eliminating these secondary effects, and the subject will be discussed by Harding and one of us in another paper; in fact we do not see at the moment any satisfactory way of disentangling the contributions to μ made by the dipole moment of the film molecules themselves and by the re-orientation of the ions and water molecules. This difficulty does not appear, however, to invalidate the above arguments as to the orientation of the dipoles in the molecules; for it is clear that, since the re-orientation of water and redistribution of ions are secondary effects consequent on the introduction of the dipoles of the film molecules at the surface, the total value of μ, including the secondary effects, will increase as the vertical component of the dipole moment of the molecule itself increases.

Confirmatory evidence of the view that the molecules are lying flat in the gaseous state and are tilted at smaller areas, is found in the fact that the pressure required to bring about the transition from gaseous to condensed film is greater with the triacetyl compound than with the diacetyl methyl ether. It is known that acetyl groups have a stronger adhesion to water than methyl ether groups; the acetates of long-chain alcohols form stable films, while the methyl ethers do not [Adam and Dyer, 1924]. The second anchorage, that of the phenolic hydroxyl, would therefore be expected to leave the water surface more easily when methylated than when acetylated. Such a re-orientation as that described above would require the phenolic polar group to leave the water surface. A re-orientation of molecules lying flat, under lateral compression, so that one of two anchorages to the water is broken, is not unknown; it has already been found with several of the long-chain dibasic acids and esters, which have two polar groups, one at each end of the molecule [Adam and Jessop, 1926, 2].

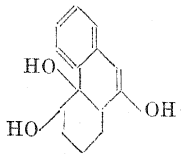
It is clear, since the molecules so easily assume the flat position, that the phenolic hydroxyl is situated near the opposite end of the molecule from the two alcoholic hydroxyls. Butenandt *et al.* [1932] favour the structure (1) or (2). These two structures seem quite impossible when the evidence of surface films is considered, since both (1) and (2) at their areas of closest packing would have a limiting area of about 70 sq. Å. instead of the 32·5 actually found. This small area of 32·5 sq. Å. could only be given by such a structure as (3), with the adjacent alcoholic hydroxyls well to the end of the molecule.

The absence of any effect of temperature on the surface pressure necessary to change the gaseous into the condensed film may perhaps mean that the

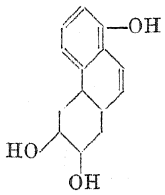
molecule is not flexible. One of us has advanced the theory [Adam, 1930, p. 75] that the change from the condensed to the expanded state, in long-chain aliphatic compounds, is due to whip-like oscillations in the flexible hydrocarbon chains. In all cases, where expansion (to either gaseous, vapour expanded, or liquid expanded films) takes place in the aliphatic series, there is a



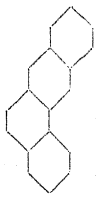
(1)



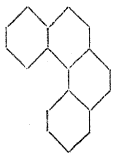
(2)



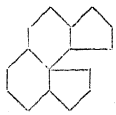
(3)



(4)



(5)



(6)

large temperature coefficient of the pressure in the transition region. The possibility of oscillations in the chains, becoming more violent as the temperature rises, naturally renders the condensed state less stable at higher temperatures. In the case of the oestrin derivatives, we have a change apparently very similar to the expansion of condensed films of aliphatic substances, but without this temperature coefficient. The most probable explanation is that the molecules are not flexible.

Evidence as to the constitution of the molecules.

Skeletons of the phenanthrene, anthracene, naphthacene, chrysene and 2 : 3-benzphenanthrene (4) types among the non-flexible structures appear admissible on the grounds of the area occupied when standing upright. The sterol skeleton of Windaus and Wieland (6) occupies about 36 sq. Å. as a minimum, as is indicated by models, and is therefore excluded. 3 : 4-Benzphenanthrene (5) is probably also too large for an area of 32·5 sq. Å. The structures of a phenanthrene type, in which the lowest ring is standing on one corner, with one of the upper ones slightly to one side, would naturally occupy an area slightly larger than that of the alkyl *cyclo*hexanols; also the anthracene and naphthacene skeletons, in which the rings are all in one line, but the lowest one is lying with a side, instead of a corner, on the water, would be somewhat larger than the *cyclo*hexanols. The inflexibility probably excludes the diphenylbenzene and phenylnaphthalene structures.

The ease with which the molecules lie flat, or stand up, as the lateral compression is changed, indicates that the phenolic hydroxyl is in a remote part

of the molecules from the two alcoholic hydroxyls. Both sets of hydroxyl groups are probably situated near the ends of the molecules. The evidence that the alcoholic hydroxyls are probably in the *cis* position, and inclined at a steep angle to the plane of the rings, has been detailed above.

Preparation of material.

Ketohydroxyoestrin monoacetate. Ketohydroxyoestrin prepared by dehydration of trihydroxyoestrin was heated to 100° for 3 hours with excess of acetic anhydride; excess of water was then added, and the precipitated acetate filtered, washed and dried. It was decolorised by boiling with charcoal in alcoholic solution, and finally recrystallised twice from aqueous methyl alcohol; M.P. 118–121°.

Trihydroxyoestrin monomethyl ether diacetate. Trihydroxyoestrin monomethyl ether was made by treating trihydroxyoestrin in alkaline solution with methyl sulphate in the cold [*cf.* Marrian and Haslewood, 1932]. 30·9 mg. of the methyl ether were heated with 5 cc. acetic anhydride at 100° for 3 hours; excess water was added, the diacetate filtered, washed and dried. It was decolorised with charcoal and crystallised once from aqueous methyl alcohol; M.P. 140–142°.

Trihydroxyoestrin triacetate. Trihydroxyoestrin was heated with excess of acetic anhydride for 3 hours at 100°; excess of water was added, and the acetate filtered off, washed and dried. The product was crystallised twice from aqueous ethyl alcohol; M.P. 119–121°.

p-Dodecyl*cyclo*hexanol and *p*-hexadecyl*cyclo*hexanol were obtained by reducing the corresponding phenols [*cf.* Adam, 1923] in alcoholic solution with hydrogen, under about 2½ atmospheres pressure, using the platinum oxide catalyst of Adams *et al.* [1928]. The products were recrystallised from alcohol.

*Dodecyl*cyclo*hexanol.* M.P. 50–55° (phenol melts at 66°). Iodine value 0. (Found: C, 80·4; H, 13·2 %; calc.: C, 80·6; H, 13·4 %.)

*Hexadecyl*cyclo*hexanol.* M.P. 65–66° (phenol melts at 79°). Iodine value 10. (Found: C, 82·3; H, 13·0 %; calc.: C, 81·6; H, 13·6 %.) The hexadecyl compound may not have been quite fully reduced.

Summary.

Surface pressure and surface potential measurements on diacetyltrihydroxyoestrin methyl ether, and triacetyltrihydroxyoestrin, show that these compounds can orient themselves at a water-air interface, either flat, or perpendicularly to the surface. The vertical component of the change in dipole moment of the surface, per molecule, due to the film, is much greater when the molecules are lying flat than when they are upright. The conclusion is drawn that the molecules probably have an anthracene or phenanthrene skeleton, or a system of four condensed rings, with two alcoholic hydroxyl groups near one end of the molecule, and one phenolic group near the other end. The

alcoholic groups are probably in the *cis* position relative to each other, on adjacent carbon atoms, and are inclined at a steep angle to the plane of the ring in which they are situated.

*Cyclo*hexanols with long chains in the *para*-position to the hydroxyl group give condensed films of area about 30 sq. Å. per molecule, slightly smaller than the cross section of the oestrin molecules.

We are indebted to the Government Grant Committee of the Royal Society and to the Medical Research Council for grants which have defrayed part of the expenses of this research.

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Note added August 17th. Since this paper was written, Marrian and Haslewood [*Lancet*, 1932, ii, 282] have shown that no double linkage other than those in the benzene nucleus is present in the oestrin molecule. This indicates that the molecule must have a four-ringed structure. Butenandt (*Nature*, August 13th) has now advanced similar views.

CXLVII. THE DETERMINATION OF SMALL QUANTITIES OF OXALIC ACID.

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(*Received June 28th, 1932.*)

The determination of small amounts of pure oxalic acid in aqueous solution by precipitation of the calcium salt and treatment of the precipitate with permanganate offers little difficulty. In the procedure elaborated by Leulier [1929] quantities of oxalic acid as low as 0·05 mg. in aqueous solution may be estimated with accuracy.

The lack of success which has attended the efforts to estimate oxalic acid in biological materials is due to the difficulty of obtaining the oxalic acid in a solution free from those substances which either inhibit the precipitation of calcium oxalate or which are carried down with the precipitate and react with the permanganate.

Investigations on the rôle of oxalic acid in metabolism have for the most part been based on the determination of oxalic acid in urine. It is not within the scope of this paper to discuss the literature in detail but a brief account of the more important investigations is given below. Salkowski [1900] estimated oxalic acid in urine by extracting the acidified urine with a mixture of alcohol and ether, afterwards precipitating the extracted oxalic acid as the calcium salt and weighing the calcium oxide formed by ignition. Autenrieth and Barth [1902] precipitated the oxalic acid directly from the urine as calcium oxalate and then transferred the oxalic acid from an acid solution into a mixture of alcohol and ether; the oxalic acid was again precipitated as calcium oxalate and the precipitate after ignition was weighed. Dakin [1907] modified the method of Autenrieth and Barth by using ether, without alcohol, in a continuous extractor. The final precipitate was either titrated with permanganate or weighed after ignition. Merz and Maugeri [1931] give references to adverse criticisms of the above methods and state that they were unable to extract oxalic acid quantitatively from its aqueous solution with ether. Van Slyke and Sendroy [1929] have described the advantages of determining small quantities of oxalic acid by a gasometric method in lieu of the many titration methods which have been used.

[1] Whole-time worker for the Medical Research Council.

Experimental.

The method of estimating oxalic acid by direct precipitation of the calcium salt from urine was found to give results both low and variable.

With regard to the extraction of oxalic acid from aqueous solution with ether, it was found that the extraction was incomplete with pure oxalic acid but with a high concentration of free mineral acid all the oxalic acid could be removed by this means. When applied to urine the ether also extracts from the strongly acidified concentrated urine many other substances, the presence of which, even though the calcium oxalate is precipitated from an alkaline, and reprecipitated from an acid, solution, may cause anomalous results.

The method about to be described depends on the fact that it was found possible to remove oxalic acid completely by esterification and distillation *in vacuo* from an acid alcoholic solution whilst apparently leaving behind all interfering substances.

Reagents. Alcohol, containing dry HCl, *N* solution. Lime-water, saturated solution, free from carbonate. Ammonium phosphate, 0·5 %.

Distillation of oxalic acid. 25 cc. of urine + 1 cc. *N* HCl are evaporated almost to dryness in a Claisen flask *in vacuo* below 50°. The flask is then connected up as shown in Fig. 1.

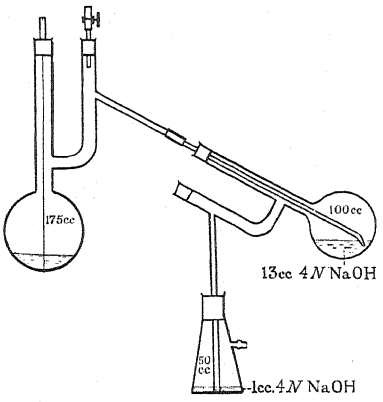


Fig. 1.

The receiver contains 13 cc. 4*N* NaOH and is kept cool by means of a rapid stream of cold water. To the residue in the flask a mixture of 5 cc. alcohol and 2 cc. benzene is added, the temperature of the water-bath surrounding the distillation flask raised to 65° and the contents of the flask dried by distilling off *in vacuo* the low boiling ternary mixture of alcohol, benzene and water. To remove the last traces of water, it is again distilled with 5 cc. acid alcohol and 2 cc. benzene.

The contents of the flask are now boiled with 20 cc. of the alcohol containing HCl on a boiling water-bath for half an hour; the flask is then reconnected to the receiver and the contents distilled *in vacuo* at 65°. The vacuum reached

about the middle of the distillation should not be less than 30 mm. The boiling and distillation are repeated in all three times.

The contents of the Büchner flask are washed into the smaller Claisen flask and the slightly alkaline solution evaporated down to 10–20 cc. *in vacuo*.

Treatment of the distillate. To free the oxalic acid from the high concentration of sodium chloride, an excess of 2 cc. 5*N* HCl is added and the solution extracted with ether in a continuous extractor for six hours. To the ether solution 5 cc. of water are added and the ether removed by distillation. The solution is filtered if necessary, made alkaline to methyl red with *N*/10 NaOH and concentrated to about 3 cc. on a water-bath.

Precipitation of the oxalic acid. The solution of oxalic acid in 3–5 cc. water in a 15 cc. centrifuge-tube is faintly acidified with *N*/10 acetic acid and mixed with 2 cc. of saturated lime-water. After standing 12 hours at 0° 0·5 cc. of ammonium phosphate is added. The precipitate is centrifuged, decanted and washed once with 2 cc. of water.

The oxalic acid in the precipitate is estimated by the gasometric method of Van Slyke and Sendroy [1929].

Results.

The following are some results on urine and on urine with added oxalic acid.

Table I.

	mg. $(COOH)_2$ found	mg. $(COOH)_2$ recovered	% error
25 cc. Urine I	0·47 } 0·45		
" "	0·43 }		
25 cc. Urine I + 1 mg. $(COOH)_2$	1·36	0·91	−9
25 cc. Urine I + 0·5 mg. $(COOH)_2$	0·96	0·51	+2
25 cc. Urine II	0·39		
25 cc. Urine II + 0·3 mg. $(COOH)_2$	0·69	0·30	0
25 cc. Urine III	0·55 } 0·56		
" "	0·57 }		
25 cc. Urine III + 0·3 mg. $(COOH)_2$	0·87	0·31	+3
25 cc. Urine III + 0·5 mg. $(COOH)_2$	1·08	0·52	+4
25 cc. Urine III + 1·0 mg. $(COOH)_2$	1·54	0·98	−2

Comparison with results obtained by ether-extraction of the urine.

250 cc. urine III were heated for twenty minutes on a boiling water-bath with 10 cc. of 10*N* HCl and concentrated *in vacuo* to 100 cc. A further 10 cc. of 10*N* HCl were added and the concentrated urine was extracted with ether for 30 hours in a continuous extractor fitted with a stirrer. After adding 5 cc. water the ether was distilled off. Strong ammonia was added to the residue to give an approximately 2*N* solution of ammonia and the contents of the flask were washed into a 15 cc. centrifuge-tube. 2 cc. *M* $CaCl_2$ were added and the precipitate left overnight, centrifuged, decanted, the precipitate washed once

with 3 cc. 8*N* ammonia and once with 3 cc. water, stirring the precipitate up with a thin rod at each washing. The precipitate was then heated with 3 cc. *N* HCl, filtered and washed with a further 2 cc. of *N* HCl, and the filtrate was diluted with a few cc. of water. The clear solution was made alkaline to methyl red with ammonia, 0·5 cc. *M* $CaCl_2$ added and *N* acetic acid till faintly acid. After standing overnight it was centrifuged, decanted, and the precipitate washed twice, each time with 2 cc. of distilled water. The precipitate was dissolved in sulphuric acid as in the Van Slyke method, and the solution transferred to a 10 cc. flask. Aliquot portions of the solution were taken for the gasometric determination.

Duplicate results gave an oxalic acid content in the 250 cc. taken of 4·9 and 5·2 mg. respectively, appreciably lower than the content as determined by distillation (Table I).

The principle of the method has also been applied to the determination of oxalic acid in blood, from which small quantities of added oxalic acid have been satisfactorily recovered. The proteins are removed by phosphotungstic acid. Further details of this work, together with a series of determinations, will be reported in a future communication.

Summary.

A method is described whereby oxalic acid can be quantitatively estimated. By a preliminary esterification and distillation the oxalic acid is freed from interfering substances and can then be precipitated as calcium oxalate and the oxalic acid in the precipitate estimated gasometrically.

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CXLVIII. A COLORIMETRIC METHOD FOR THE DIRECT DETERMINATION OF UREA IN URINE.

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(*Received June 30th, 1932.*)

Since Fosse [1914] introduced a gravimetric method for the estimation of urea in urine based on the precipitation of urea as dixanthylurea, no attempt has been made to apply the principle to meet the requirements of the routine laboratory analyst who has many estimations to perform in a limited time. The purpose of this paper is to present a method suitable for use in the routine laboratory.

The tedious process of collecting, washing and weighing the dixanthylurea has been replaced by the separation of the precipitate by centrifuging; the nitrogen content of the substance is determined by a colorimetric estimation of the ammonia produced in a micro-Kjeldahl digestion, which has been facilitated by the introduction of a modified digestion reagent containing selenium, instead of copper, as catalyst [Lauro, 1931], and perchloric acid instead of sulphuric acid as the oxidising agent. The advantages of perchloric acid are mentioned in a recent paper by King [1932], who uses this acid for the estimation of total phosphorus in blood. The whole process may be carried out in the same tube. The difficulty of sedimentation of the light flocculent precipitate of dixanthylurea has been overcome by precipitating the dixanthylurea in the presence of a very fine suspension of barium sulphate, when the two precipitates centrifuge down together leaving a clear supernatant liquid which may be safely decanted.

Proteins and interfering substances are removed from the urine by adsorption on "colloidal iron" which is produced in the liquid by the interaction of dilute ferric chloride and sodium bicarbonate solutions.

Method.

Reagents.

(1) 1 % crystalline ferric chloride in water.

(2) 0·5 % sodium bicarbonate in water.

(3) Barium reagent. Dissolve 10 g. of sodium chloride and 2 g. of sodium sulphate in 90 cc. of distilled water. Add this solution to 400 cc. of glacial

acetic acid, and to the mixture add 1·2 g. of barium chloride dissolved in 10 cc. of water.

(4) 2·5 % solution of xanthydrol (Schering-Kahlbaum) in glacial acetic acid. This solution should be kept in a small well-stoppered bottle and prepared every few days.

(5) Digestion mixture:

100 cc. of 60 % perchloric acid;
40 cc. of phosphoric acid (sp. gr. 1·75).
20 cc. of 3 % sodium selenate solution;
0·5 cc. of 10 % sodium sulphate solution.
130 cc. of water.

(6) Nessler reagent [Folin and Wu, 1919].

(7) Ammonium sulphate solution. Dissolve 0·1179 g. of pure ammonium sulphate in water. Preserve from moulds with two drops of strong sulphuric acid and dilute to 1 litre.

Procedure. Measure 1 cc. (or 2 cc. if the urea content is very low) of urine from an Ostwald pipette into a 100 cc. graduated flask, add 2 cc. of 1 % ferric chloride and, while rotating the liquid, 10 cc. of 0·5 % sodium bicarbonate solution. Dilute to the mark, mix and filter. Transfer a suitable amount of filtrate (1–5 cc. depending upon the urea content) with an Ostwald pipette to a 4″×1″ hard glass round-bottomed centrifuge-tube[1] of 35–40 cc. capacity. Table I has proved useful in choosing the volume of filtrate required, but it

Table I.

Specific gravity of specimen	cc. of filtrate required
1030–1025	1
1025–1020	1·5
1020–1015	2
1015–1010	3
1010–1005	5
Below 1005	Use 2 cc. of urine for filtration

must be borne in mind that other factors beside the urea content affect the specific gravity of a urine.

Dilute the filtrate in the centrifuge-tube to 5 cc. with water, add 5 cc. of well mixed barium reagent and 1 cc. of xanthydrol solution. Mix the liquid, and allow the tube to stand in a bath of ice-cold water for 20 minutes. Mix the liquid by rotating the tube; wash down the sides of the tube with 1 cc. acetone, which is allowed to float on the surface. Centrifuge for five minutes and decant the supernatant liquid, placing the inverted tube on a sheet of filter-paper for two minutes to drain. Remove the excess liquid from the inside of the tube by wiping with filter-paper.

Add 1 cc. of digestion mixture and a piece of porous pot. Heat the tube gently on a sand-bath in a fume chamber, covering the mouth of the tube with a watch-

[1] Supplied by C. Dixon and Co., 27 Devonshire Street, W.C. 1.

glass when heavy white fumes appear and the liquid darkens. Twenty seconds after the brown-coloured liquid becomes colourless remove the tube from the sand-bath. Add 20 cc. of water after the acid has cooled for a few minutes. Cool to room temperature and add 7·5 cc. of Nessler reagent while twirling the liquid in the tube. After mixing, centrifuge for three minutes to remove the precipitate of barium sulphate. Compare the crystal clear supernatant liquid with the standard which is prepared at the same time as follows. Place 5 cc. of barium reagent and 5 cc. of water *etc.* in a 4″×1″ tube, and centrifuge, drain and digest as described for the unknown. After heating, add 15 cc. of water and 5 cc. of standard ammonium sulphate solution before adding 7·5 cc. of Nessler reagent.

The use of a thick blue glass light filter for the comparison in the colorimeter is recommended, since by this means the difficulty always associated with the matching of yellow or brownish-yellow colours is eliminated; the red tints which result from the use of the blue glass being easily compared.

Calculation (if the standard is set at 20 mm.):

$$\frac{53{\cdot}6}{\text{Colorimetric reading} \times \text{number of cc. filtrate used}} = \text{g. of urea per 100 cc. of urine.}$$

Table II, which contains results taken at random from many analyses, gives the experimental values found by the analysis of standard urea solutions.

Table II.

Concentration of solution taken in g. per 100 cc. %	Experimental result in g. per 100 cc. %	Percentage error
4·2	4·23	+1
3·0	3·05	+1·5
2·8	2·73	−2·5
2·0	1·98	−1
1·5	1·48	−1
1·0	0·98	−2
0·75	0·73	−2·5
		Mean error 1·6

The recovery of urea added to urine is shown in Table III.

Table III.

% urea in urine	% urea added	Total urea %	% urea found
0·81	1·0	1·81	1·82
0·65	1·0	1·65	1·67
0·85	1·5	2·35	2·32
4·21	0·75	4·96	5·00
0·29	1·5	1·79	1·75
1·58	1·0	2·58	2·55

The close agreement between the xanthydrol and urease methods when applied to a number of specimens of urine is shown in Table IV. The direct titrimetric method of Cole [1931] was employed for the estimation of urea by the urease method.

Table IV. *Comparison of results obtained by urease and xanthydrol methods.*

Result by xanthydrol method (g./100 cc.)	Result by urease method (g./100 cc.)
1·55	1·56
1·19	1·18
0·54	0·55
1·53	1·48
0·57	0·56
1·57	1·55
2·31	2·27
2·80	2·81
3·15	3·08
0·72	0·71
2·76	2·78
1·38	1·35

Discussion.

Twelve urines were examined in order to demonstrate the absence of adsorption of any nitrogen-containing substance on the barium sulphate. To illustrate this, thirty times the amounts of reagents and urine filtrate normally taken were used, xanthydrol not being added. After the precipitate of barium sulphate was spun down and subjected to the usual treatment, no appreciable colour developed when the liquid was treated with Nessler reagent.

The digestion is free from difficulty, as owing to the small volume of liquid which has to be removed by boiling, bumping is practically unknown. The end of the digestion is clearly defined, the liquid changing from brown to colourless in a few seconds.

It is unnecessary to wash the precipitate of dixanthylurea as the amount of urea-free liquid left in the tube after the treatment described is of the order of 0·1 cc. If desired, the tubes may be calibrated at 30 cc. and the contents of the tube diluted to this mark, but this is not essential.

The ferric chloride and sodium bicarbonate solutions when used in the amounts stated will remove up to 2·5 % of protein from urine. That no adsorption of urea takes place during the precipitation was shown by treating a mixture of 1 cc. of 0·6 % urea solution and $\frac{1}{3}$ cc. of plasma (containing 40 mg./100 cc. of urea) with the protein precipitants before diluting to 100 cc. The urea content of the filtrate was found to be 0·0061 %.

We are at present engaged in applying the principle to the determination of urea in blood-filtrates.

Summary.

A method is described for the routine estimation of urea in urine based on the formation of dixanthylurea. Interfering substances are removed from the urine with ferric chloride and sodium bicarbonate, and the urea is precipitated from the filtrate by xanthydrol. The precipitate is centrifuged in the presence of barium sulphate and its nitrogen content determined colorimetrically after a micro-Kjeldahl digestion.

We wish to express our thanks to Prof. E. C. Dodds for his help and advice concerning a number of problems which arose in the course of this work, and to Dr T. Bloem for his kind assistance.

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CXLIX. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

VI. THE CONVERSION OF FRUCTOSE AND GLUCOSE TO LACTIC ACID BY EMBRYONIC TISSUES.

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(*Received June 30th, 1932.*)

FRUCTOSE is known to be utilised by certain animal and vegetable cells; thus it is fermented at least as rapidly as glucose by yeast-juice [Harden and Young, 1908; 1909] and certain yeasts [Slator, 1908; Willstätter and Sobotka, 1922; Fernbach, Schoen and Mori, 1927; Hopkins, 1931]; it is converted into lactic acid by tumours though to a less extent than glucose [Minami, 1923; Warburg, Posener and Negelein, 1924; Rosenthal, 1930, 1]; whilst in the summer months minced frog's muscle [Laquer and Meyer, 1923] and rat-liver [Rosenthal, 1930, 2] form lactic acid from fructose more rapidly than from glucose. On the other hand, brain tissue [Loebel, 1925] and nerve tissue [Gerard and Meyerhof, 1927] cannot convert fructose into lactic acid. The action of the other tissues has apparently hitherto not been investigated. Some preliminary experiments by the authors, however, indicate that with kidney, spleen, testis and retina there is little anaerobic attack on fructose.

Whilst fructose is not in general present in any considerable amount in the body, it has been known since the time of Claude Bernard that fructose normally occurs in the pregnant animal of several species, attaining a high concentration (up to *ca.* 1 %) in the amniotic and allantoic fluids and in the foetal blood [Bernard, 1850; Dastre, 1876; Gürber and Grünbaum, 1904; Paton, Watson and Kerr, 1907; Orr, 1924]. Therefore, it becomes of special interest to find out if fructose is utilised by embryonic tissues, which are in many cases bathed in a medium rich in this sugar. In the present paper the anaerobic conversion of fructose and glucose into lactic acid by the different embryonic tissues of several species is described.

The work of Warburg and his collaborators [Warburg, 1926, 1930] has shown the extraordinary ability of embryonic tissues to form lactic acid from glucose, the foetal membranes of the rat resembling the most active tumours in this respect. It may be noted, however, that almost all these investigations refer to the rat, and therefore the figures which we give for other species are of interest.

It will be shown that while glucose is converted readily into lactic acid by all the embryonic tissues, the attack on fructose depends both on the tissue and on the species, varying from zero to a value equal to that observed with the same tissue on glucose.

The discussion of the results follows the experimental section.

Nomenclature. For the sake of brevity, we shall use the following terms.

Glycolysis.	General expression denoting the breakdown of carbohydrate to lactic acid [*cf.* Warburg, 1925].
Glucolysis.	The glycolysis in the presence of added glucose.
Fructolysis.	The glycolysis in the presence of added fructose.
Autoglycolysis.	The glycolysis when no substrate is added.

This distinction between glycolysis and glucolysis is in conformity with the officially approved nomenclature now adopted for the glycosides [Armstrong and Armstrong, 1931].

EXPERIMENTAL.

Preparation of material and methods used.

The smaller animals were killed by a blow on the neck; the larger ones were killed under ether anaesthesia. The animal was immediately immersed in warm, dilute lysol, and after a few minutes was washed thoroughly with running water. The uterus was then removed and dissected with sterile instruments.

After dissection, portions of the embryonic membranes of suitable size were cut with scissors: such material is particularly suitable for *in vitro* experiment since it is sufficiently thin to be used directly without further cutting. The placenta was used intact when it was very small, otherwise it was cut with a razor into thin slices. In all cases the material was rinsed in a dish containing Ringer solution before being transferred to the manometer vessels, where the tissues were suspended in Ringer solution containing 25 *mM.* per litre of sodium bicarbonate and saturated with nitrogen containing 4·5–5 % CO_2 by volume (p_H 7·45–7·50). Sugar was added to the Ringer solution in freshly prepared 10 % solution to give in all cases a final concentration of 0·2 %. The fructose used was Schering-Kahlbaum "Laevulose cryst. for scientific purposes." Recent analyses of this product have been made by Wierzuchowski *et al.* [1931]; polarimetric measurements on the specimen used by us agree with those given by these authors.

The glycolysis was measured manometrically at 38° by the method of Warburg [1926; 1930]. The first readings were taken 10 to 15 minutes after putting the vessels in the bath, and were usually continued for a period of 2 to 3 hours. These prolonged experiments were desirable since we have found that the autoglycolysis of embryonic tissues is often large in the first hour of the experiment. The general procedure was to make simultaneous observations of the glycolysis of pieces of the same tissue in glucose, fructose and sugar-free Ringer solutions.

In typical cases (rat-yolk-sac and cat-amnion) the lactic acid formation

measured manometrically was confirmed by chemical estimation by Clausen's method. The results show that the action of the embryonic membranes on fructose is a pure lactic acid fermentation.

The glycolysis is expressed throughout by Warburg's quotient $Q_M^{N_2}$, which is defined as the number of mm.^{3} of CO_2 at N.T.P. liberated per hour per mg. dry weight of tissue. Since 1 gram-equivalent of lactic acid liberates 22·4 litres of CO_2, the lactic acid per hour formed is expressed as a percentage of the dry weight of the tissue when these figures are multiplied by 0·4.

The disposition and development of the embryonic membranes in the several species are so varied that in the following text we quote references to standard works for the description of the individual membranes as they arise. The experiments are numbered with respect to the individual animals; experiments bearing the same number thus refer to tissues of the same animal throughout.

Glycolysis of the foetal membranes.

Outer sac.

The results are collected in Table I.

Although for the sake of convenience we describe together the outer membranes of the various species, it must be remembered that the outer sac of Rodentia consists of the inverted inner half of the yolk-sac, the outer half of the yolk-sac having disappeared together with the greater part of the chorion laeve during the second half of pregnancy [Grosser, 1927, p. 122]. On the other hand, the outer sac of Carnivora [Grosser, 1909, pp. 131–2] consists of the chorion laeve in close contact with the outer wall of the allantois.

Rodentia.

Rat (*albino*). (For description of membranes see Grosser [1927, p. 135].) The inner and outer membranes were dissected from the embryo and placenta, and the yellowish yolk-sac carefully freed from the fine white amnion. Pregnancies of known date are readily obtained in this species from observations of the occurrence of vaginal plugs. Observations on the glucolysis, fructolysis and autoglycolysis of the yolk-sac of rats 13 to 20 days pregnant (*i.e.* in the second half of pregnancy) are given. The glucolysis remained practically constant for the 3 hours of the experiment, the Q values agreeing well with those obtained in Ringer solution by Kumanomido [1928]. The fructolysis is of the same order of magnitude as the glucolysis (in Exp. 7 the values were identical for the 2 hours of the experiment), but in no case did the fructolysis exceed the glucolysis. In Exps. 4 and 7 the fructolysis and glucolysis ran parallel, in the remainder the fructolysis fell off more rapidly than the glucolysis. The autoglycolysis was sometimes appreciable in the first 30 minutes, but afterwards fell to a negligible value.

Mouse [Jenkinson, 1913, p. 232]. Whilst the glycolysis of the mouse-yolk-

Table I. *Outer membrane.* $Q_M^{N_2}$.

Exp. no.	Glucolysis 1	Glucolysis 2	Glucolysis 3	Fructolysis 1	Fructolysis 2	Fructolysis 3	Autoglycolysis 1	Autoglycolysis 2	Autoglycolysis 3	Embryo Wt. g.	Embryo Age days	Relative fructolysis 2nd hr. %
	hours			hours			hours					
Rat (albino)												
4	35·5	32·5	31·5	27·0	26·5	27·5	1	0	0	0·065	13	82
3	32·0	27·5	27·0	30·0	22·0	20·0	4	1	0·5	0·175	15	79
				28·0	22·0	19·0	—	—	—	—	—	79
				30·0	22·0	—	—	—	—	—	—	79
8	24·2	24·0	21·4	21·2	18·8	13·7	—	—	—	0·34	16	—
35	18·0	17·0	17·5	11·5	9·0	8·5	4	1	1	2·6	20	50
1	24·0	19·0	18·0	18·0	13·0	11·0	7	1·5	0	3·2	20	66
7	19·0	16·0	—	20·0	15·5	—	2·5	0	—	3·2	20	97
				20·0	15·0	—	—	—	—	—	—	94
				20·0	16·0	—	—	—	—	—	—	100
											Mean	75
Mouse												
27	26·2	22·8	—	7·0	4·0	—	7·2	2·3	—	0·425	15	8
33	24·2	20·3	—	5·8	3·7	—	6·7	1·1	—	0·62	16	13·5
42	26·0	19·0	—	9·5	4·5	—	9·0	2·0	—	1·0	17	15
											Mean	12
Guinea-pig												
18	11·2	*11·2*	—	11·9	*11·6*	—	8·4	*3·2*	—	30	50	105
29	11·0	11·0	—	10·0	9·5	—	2·0	0·5	—	31·5	50	86
											Mean	96
Rabbit												
26	25·5	25·0	—	12·0	10·0	—	6·0	1·0	—	0·07	13	37
24	17·0	15·0	—	9·5	7·5	—	4·5	1·0	—	30·2	27	46
22	22·4	23·1	—	5·5	4·4	—	3·0	1·1	—	31·6	27	15
											Mean	33
Cat												
11	*41·0*	*24·0*	—	*6·0*	*5·0*	—	*5·0*	*5·0*	—	19·5	—	0
38	16·5	13·5	13·0	3·0	2·5	1·5	2·0	1·0	1·0	45·5	—	12
13	17·0	15·0	—	4·8	2·8	—	2·9	1·8	—	46·0	—	7·5
36	17·0	14·4	—	4·1	2·2	—	3·3	1·4	—	58·5	—	6
10	17·0	10·5	—	3·0	2·5	—	3·5	2·0	—	109	—	6
											Mean	6
Ferret												
23	34·5	31·5	—	—	—	—	4·0	1·3	—	1·4	—	—
19	*29·0*	*27·0*	—	*6·0*	*5·0*	—	*6·0*	*4·0*	—	2·9	—	4
34	29·0	23·0	19·5	8·0	7·0	6·0	3·0	0·5	0·3	6·0	—	29
				8·5	6·0	5·5	—	—	—	—	—	24
											Mean	15·5
Human peripheral membrane												
Maternal side												
17	9·3	8·0	—	6·3	4·6	—	2·9	0·8	—	10 months		53
21	8·0	6·3	—	4·2	2·4	—	2·3	1·0	—	—	—	26
28	8·2	7·9	—	7·2	6·0	—	2·1	0·8	—	—	—	73
											Mean	51
Foetal side												
17	3·8	1·9	—	4·1	0·6	—	3·8	0	—	—	—	32
20	3·9	*3·0*	—	3·6	*2·6*	—	2·5	*1·4*	—	—	—	75
28	6·5	6·0	—	4·3	2·9	—	1·2	0·7	—	—	—	42
											Mean	50

The figures given in italics in these Tables are the values of $Q_M^{N_2}$ calculated from half-hour experimental periods. The ages given are calculated from the weights, using the tables of Needham [1931, Appendix I]: in the rats the ages were known, and agreed with those calculated.

sac is shown to be comparable with that of the rat, the fructolysis on the other hand is negligible, barely exceeding the autoglycolysis.

Guinea-pig [Grosser, 1927, pp. 38, 148]. Values for two late pregnancies are given. Although the glucolysis and fructolysis are less than those of the rat at a correspondingly late stage (Exps. 1 and 7, Table I), the fructolysis resembles that of the rat in being practically identical with the glucolysis.

Rabbit [Grosser, 1927, p. 122]. Results for two very advanced pregnancies and for one half-way through gestation are included in Table I. The glucolysis is of the same order as that of the rat-yolk-sac, but the membrane differs from the yolk-sac of the other rodents investigated in that it attacks fructose at about one-third of the rate at which it converts glucose to lactic acid. The fructolysis as well as the glucolysis remains constant over the experimental period.

Carnivora.

Cat. The allanto-chorion of the cat was used without attempting to separate it into its component layers, although it was noted that such a separation occurred partially during the course of the experiment. The membrane is not inferior to that of the rat in attacking glucose, but the fructolysis is negligible, being only slightly greater than the autoglycolysis.

Ferret. The allanto-chorion of the ferret was used in the same way as that of the cat. The glucolysis is at least as high as that of the rat; the fructolysis, while steady and relatively greater than that of the cat, is still small.

Man. The human material used in this paper was obtained from the full-time afterbirth only, and was used within half an hour of its expulsion, while the placenta was still warm. It was kindly supplied by the Obstetric Department of the Middlesex Hospital.

The membrane attached peripherally to the placenta of the afterbirth consists, after separation of the amnion, of chorion laeve together with decidua [Grosser, 1909, pp. 269, 212]. As this membrane was thick, it was divided. On pulling apart, it separated fairly easily along a plane nearer to the foetal side into foetal and maternal portions, which were used separately in the manometers. Histological examination indicated that the cleavage had occurred through the "Zwischenzone" [Grosser, 1909, p. 212] which consists of chorionic epithelium and the remains of villi, decidua capsularis and decidua compacta. The foetal portion thus consisted largely of chorionic connective tissue together with a small amount of the "Zwischenzone"; the maternal part consisted largely of the latter. As was to be expected from the known low metabolic activity of connective tissue, it was found (Table I) that the metabolism of the maternal portion was higher than that of the foetal. Both attacked fructose, but to about half the extent to which they attacked glucose.

Amnion.

The glycolysis of the amnion of the rat, rabbit, guinea-pig, cat and ferret in glucose, fructose and sugar-free solutions is shown in Table II. The preparation of the membrane presented no difficulties; in the rabbit, cat and

Table II. *Amnion.* $Q_M^{N_2}$.

Exp. no.	Glucolysis 1 hours	Glucolysis 2 hours	Glucolysis 3 hours	Fructolysis 1 hours	Fructolysis 2 hours	Fructolysis 3 hours	Autoglycolysis 1 hours	Autoglycolysis 2 hours	Autoglycolysis 3 hours	Embryo Wt. g.	Embryo Age days	Relative fructolysis 2nd hr. %
Rat (albino)												
32	16·0	16·0	16·0	5·0	3·0	2·0	6·0	0	0	1·6	19	19
35	15·0	14·5	14·5	7·0	3·5	4·0	—	—	—	2·6	20	Not>24
7	17·0	11·5	—	7·0	2·0	—	—	—	—	3·2	20	Not>17
												Mean 19
Rabbit												
37	17·5	13·5	14·0	5·5	4·5	4·5	1·5	0·5	0	36·5	28	31
				6·5	5·0	5·5	—	—	—	—	—	35
												Mean 33
Guinea-pig												
18	5·1	*5·2*	—	3·8	*2·6*	—	2·8	*0·2*	—	30·0	50	48
29	5·8	6·0	—	4·0	2·5	—	2·6	0·4	—	31·5	50	38
												Mean 43
Cat												
11	*17·0*	*11·0*	—	*10·0*	*8·0*	—	*4·0*	*4·0*	—	19·5	—	57
13	15·7	15·0	—	6·9	7·5	—	3·8	1·9	—	46·0	—	43
41	—	—	—	10·6	8·2	8·0	—	—	—	57·0	—	—
				14·6	10·3	9·8	—	—	—	—	—	—
36	12·5	12·5	—	6·5	7·5	—	6·0	4·5	—	58·5	—	37
												Mean 46
Ferret												
23	15·5	13·5	*13·0*	13·5	13·0	*12·0*	10·5	1·0	0	1·4	—	96
19	*18·4*	*13·2*	—	*12·8*	*10·4*	—	*8·2*	*1·6*	—	2·9	—	76
												Mean 86
Rabbit-allantois												
24	22·0	14·2	—	16·0	11·5	—	12·0	2·5	—	30·2	27	77
37	19·0	20·0	20·0	13·0	11·5	11·5	1·0	0·5	0	36·5	28	56
				12·0	12·5	11·5	—	—	—	—	—	62
												Mean 68
Cat-yolk-sac												
38	21·0	19·5	17·5	11·5	9·5	8·5	5·0	2·5	1·5	45·5	—	41
				11·0	9·5	10·5	—	—	—	—	—	41

ferret, dorsal portions were taken in order to avoid free allantois. The membrane in the cat and ferret consists of fused allantois and amnion [Grosser, 1909, p. 132]. The glucolysis was in all cases steady over the course of the experiment. It should be noted that the values obtained for the rat were from late pregnancies, and therefore were lower than the value in horse-serum obtained from an early pregnancy by Negelein [1925]. The fructolysis of amnion of the rat is small compared with the glucolysis; that of the ferret approaches the glucolysis; whilst in the rabbit, guinea-pig and cat the amnion is intermediate in its activity towards fructose.

The metabolism of the amnion from the human afterbirth at term was

found to be very low. This is scarcely surprising since the amnion undergoes a hyaline degeneration at the end of pregnancy, which Belosor [1925] considers to be a physiological preparation for its rupture.

Allantois.

Measurements on the allanto-chorion and allanto-amnion of the Carnivora have been described above. Free allantois can be obtained in sufficient quantity from the rabbit. In the later stages the allantoic sac stretches between the placenta and the ventral surface of the embryo, over which it is fused with the amnion [Grosser, 1927, p. 129]. The side portions consist of pure allantois and are easily dissected. The results are given in Table II, from which it will be seen that the glucolysis is high and almost identical with that of the yolk-sac of the same animal (Exp. 24). The allantois readily attacks fructose, to a greater extent than either the amnion or the yolk-sac of the same animal. The good agreement between the duplicates in fructose shows that the results are highly reproducible and the membrane homogeneous.

Yolk-sac.

The modified yolk-sac of Rodentia, which takes on the special nutritive function of the primary chorion laeve [Grosser, 1927, p. 84] and even develops villi in the later stages [Robinson, 1892], has been studied above. In Carnivora, the yolk-sac persists throughout pregnancy as a small intact sac, presumably with very different function from that of the rodents. The cat was the most convenient source of this type of yolk-sac. In this animal it is found as a yellow sac lying between embryo and placenta and drawn out into two long horns in the direction of the long axis of the uterus. It was easily dissected and freed from allantois. The results are given in Table II. It was found to attack glucose slightly more readily than the allanto-chorion or allanto-amnion from cat embryos of the same age. The fructolysis was a little less than half the glucolysis, and remained constant. Here again the uniformity of the material is shown by the equal fructolysis of two portions from different parts of the membrane.

PLACENTA.

Previous studies have shown that the placenta of the rat [Murphy and Hawkins, 1925; Loeser, 1927], mouse [Fujita, 1928], rabbit [Bell *et al.*, 1928] and man [Loeser, 1927; Bell *et al.*, 1928; Kumanomido, 1928] readily converts glucose into lactic acid. These workers all used either the whole organ or slices cut from it; placenta *Brei* has been studied by Ishikawa [1928] (rabbit) and Hayashi [1928] (human), who differ as to whether added hexoses are converted into lactic acid at all by the placenta.

We have studied the glucolysis, fructolysis and autoglycolysis of the placenta of the rat, mouse, guinea-pig, rabbit, cat, ferret and human being (Table III).

Table III. *Placenta*. $Q_M^{N_2}$.

Exp. no.	Glucolysis 1	2	3	Fructolysis 1	2	3	Auto-glycolysis 1	2	3	Embryo Wt. g.	Age days	Relative fructolysis %	Remarks
	hours			hours			hours						
Rat													
16	*17·2*	*12·8*	—	*12·8*	*5·6*	—	*9·8*	*5·2*	—	0·03	13–14	5	Whole placenta
8	13·2	11·3	11·7	6·5	3·5	1·6	4·1	2·9	1·8	0·34	16	7	—
32	19·5	14	14	8	3	2·5	7·5	1·5	2	1·6	18–19	12	—
1	16·5	15·5	15	4·0	2·5	2·0	3·5	2·0	2·0	3·2	20	4	—
											Mean	7	
Mouse													
27	7·0	6·5	—	4·8	3·4	—	5·3	3·1	—	0·425	15	9	Materna half
27	8·3	8·0	—	2·6	2·1	—	3·5	2·0	—			2	Foetal half
33	7·2	7·3	—	2·4	1·7	—	2·7	1·3	—	0·62	16	7	—
											Mean	6	
Guinea-pig													
18	5·1	*4·2*	—	3·3	*1·4*	—	2·5	*1·2*	—	30	50	7	—
29	4·25	3·15	—	2·1	0·9	—	1·8	0·8	—	31·5	50	4	—
											Mean	6	
Rabbit													
26	9·6	7·2	—	4·5	2·2	—	6·6	4	—	0·072	14	0	—
24	4·6	3·4	—	1·9	1·5	—	1·95	1·0	—	30·2	27	21	—
22	4·6	3·9	—	2·3	1·5	—	1·7	0·9	—	31·6	27	20	—
											Mean	14	
Cat													
11	*6·0*	*5·6*	—	*3·8*	*3·0*	—	*2·6*	*1·4*	—	19·5	—	26	—
40	7·2	5·8	5·6	4·5	3·4	3·3	2·6	0·9	0·8	—	—	51	Foetal
	3·8	3·0	3·1	5·0	3·6	3·4	1·8	0·4	0·3	69	—	(120)	Mid
	5·2	4·5	3·8	3·4	2·2	1·6	1·0	0	0	—	—	49	Maternal
43	4·4	4·1	3·8	2·4	1·6	1·6	1·0	0·7	0·6	—	—	26	Foetal
	3·6	3·1	3·2	2·3	1·5	1·4							
	5·3	4·5	4·4	2·8	1·8	1·5	1·5	0·6	0·6	85	—	33	Mid
	4·4	3·7	3·6	2·0	1·4	1·0	1·0	0·4	0·4	—	—	30	Maternal
10	6·4	5·6	—	4·1	2·5	—	2·2	0·6	—	109	—	38	—
											Mean	36	
Ferret													
23	22	14	—	8·3	5·5	—	5·4	1·5	—	1·4	—	32	—
19	*21·4*	*19·8*	—	*8·8*	*6·2*	—	*4·7*	*1·4*	—	2·9	—	26	—
											Mean	29	
Human													
0	3·7	2·6	—	3·6	2·1	—	1·6	0·4	—	—	—	77	Maternal
28	5·7	4·5	—	4·7	2·9	—	1·7	0·4	—	—	—	61	Maternal
28	4·0	2·5	—	2·3	0·9	—	1·3	0·4	—	—	—	24	Foetal
17	4·6	4·0	—	4·5	2·8	—	—	—	—	—	—	—	Foetal
21	2·9	1·5	—	2·5	1·3	—	2·3	0·6	—	—	—	62	Foetal
				2·5	1·0								
											Mean	57	

Rodentia (placenta haemochorialis labyrinthica).

Although the placentae examined were from different stages of pregnancy, inspection of the figures shows that the glucolysis is highest in the case of the rat, the other three animals having much lower Q values. In Exp. 16 whole placentae were used; in Exp. 27 the placenta was divided horizontally into maternal and foetal halves which agreed with one another and with the slices of Exp. 33 in their metabolism.

The placentae of the four rodents hardly attacked fructose, the fructolysis being little larger than the autoglycolysis.

Carnivora (placenta endotheliochorialis).

The placenta of the cat is zonary, that of the ferret zono-discoidal [Strahl, 1906, p. 301] consisting of two discs separated on the antimesometrical side by a blood extravasation. The ferret placenta showed a very high glucolysis: fructose was also attacked but only to about one-third the extent of glucose. In the cat the glucolysis was lower, but fructose was attacked relatively to about the same extent as in the ferret.

As both maternal and foetal tissues enter into the placenta of Carnivora, we have compared the activity towards glucose and fructose at different depths through the placenta (Exps. 40 and 43, Table III). The placenta was divided horizontally into three portions, after rejecting thin layers from the outer surfaces. Slices were cut from the foetal, middle and maternal portions thus prepared and were used separately for manometric measurements. Histological examination of the portions used in Exp. 40 showed that whilst the foetal and mid parts consisted entirely of placental labyrinth, the maternal portion contained labyrinth together with the mixed maternal and foetal tissues of the "invasion-zone" [Grosser, 1909, p. 128; 1927, p. 119]. The glucolysis remains approximately constant as we pass through the placenta, although an apparently anomalous low result was obtained for the middle portion in Exp. 40. The fact that the duplicates in Exp. 43 show rather a high divergence illustrates the variable nature of the material, which makes accurate comparison difficult. The fructolysis falls off with the autoglycolysis as the maternal side is approached.

Man (placenta haemochorialis olliformis).

Full-time human placentae were obtained as already described. Slices were cut from the warm placenta, the larger white infarcts being avoided and the surface layers rejected. The Q values for full-time human placentae show a greater variation among themselves than that shown by the placental tissue of the other animals; this is no doubt due to the large amount of metabolically inert fibrinoid tissue in the human placenta. There was a relatively large autoglycolysis in the first hour, which fell to a small value in the second. In all cases the glucolysis was considerably higher than the autoglycolysis, particularly in the second hour. The figures given show no systematic difference between the metabolism of maternal and foetal sides. Both sides definitely attack fructose but to a less extent than glucose, the fructolysis falling off rather more rapidly than the glucolysis. These experiments show clearly, in contradistinction to those of Hayashi [1928] on *Brei*, that the human placenta converts glucose more readily than fructose to lactic acid, under our experimental conditions.

Embryo and foetal tissues.

Embryos were taken of a size suitable for use without cutting, and were carefully freed from membranes. Table IV shows that the rat embryo of the

Table IV. *Embryo and foetal tissues.* $Q_M^{N_2}$.

Exp. no.	Glucolysis 1 hour	Glucolysis 2 hours	Glucolysis 3 hours	Fructolysis 1 hour	Fructolysis 2 hours	Fructolysis 3 hours	Autoglycolysis 1 hour	Autoglycolysis 2 hours	Autoglycolysis 3 hours	Embryo Wt. Dry wt. mg.	Embryo Age days	Relative fructolysis %
						Embryo.						
Rat												
14	14	10	—	4	2·5	—	3	1·5	—	3·4	13	10
30	—	—	—	3·9	2·5	1·8	4·6	1·8	0	3·8	13	(7)
5	—	—	—	4·6	2·2	2·6	2·8	1·6	1·3	4·7	13	(6)
16	—	—	—	3·7	—	—	3·9	—	—	5·3	13–14	—
Rabbit												
26	15·9	12·9	11·2	2·8	1·4	1·3	—	—	—	12·0	14	11
Chick												
9	15·5	—	—	—	—	—	—	—	—			
	12·0	—	—	—	—	—	—	—	—	3·8	4	—
	22·5	—	—	2·3	—	—	1·7	—	—			(4)
15	—	—	—	3·5	1·2	—	2·9	1·2	—	5·2	4½	0
2	—	—	—	3·0	1·0	1·0	2·5	1·0	1·0	10·5	5	0
6	—	—	—	3·6	0·8	0·4	3·6	0·8	0·4	10·7	5	0
						Foetal tissues.						
Cat-liver										Moist wt. g.		
11	5·2	—	—	2·6	—	—	—	—	—	19·5	—	—
Guinea-pig-liver												
18	*3·4*	*2·6*	—	*2·6*	*1·8*	—	*2·0*	*0·6*	—	—	50	60
Mouse-liver												
42	12·5	9·6	8·9	10·4	9·2	8·8	11·6	10·2	8·3	1·0	17	—
Mouse-skin												
42	18·5	17·0	16·5	6·5	3·0	2·5	6·5	3·5	2·0	1·0	17	0
Cat-lung												
41	17	14	—	4·0	3·5	—	4·5	2·5	—			
Cat-brain										57	—	0
41	*19*	*15*	—	*2·4*	*2·0*	—	*1·8*	*1·4*	—			4

13th to 14th day and the rabbit embryo of the 14th day do not fructolyse. These embryos are in the mid-period of gestation. The chick embryo of 4 to 5 days' incubation shows a similar inability to attack fructose.

For the investigation of the later embryo, the large size necessitates the use of individual tissues. The experiments on the foetal brain, liver, lung and skin given in Table IV were designed to find out if these tissues develop any special fructolytic power in the terminal stages of gestation. The liver of the cat and guinea-pig was used in slices; that of the mouse was small enough to be used as a whole organ. In the guinea-pig there was a definite attack on glucose (contrast adult rat-liver [Rosenthal, 1930, 2]); and the fructolysis lay

between the glucolysis and autoglycolysis. In the mouse all three coincided, the autoglycolysis being extraordinarily high. The skin of the mouse embryo and slices of embryonic brain and lung of the cat, all failed to attack fructose, though their glucolysis, like that of most embryonic tissues, was high.

Chemical estimation of lactic acid formed from fructose.

The manometric measurements of the fructolysis in two representative cases, rat chorion and cat amnion, were controlled by simultaneous lactic acid determinations by Clausen's method. For this purpose the contents of the vessel were removed and acidified immediately after taking the last manometer-reading; the proteins were precipitated by tungstic acid, and sugar was removed from the filtrate by the copper-lime reagent. The filtrations were carried out through Jena glass filters. The lactic acid was determined by the method of Friedemann and Kendall [1929]; instead of aerating, however, washed carbon dioxide from a cylinder was used. Duplicate specimens of tissue were placed in the bath at the same time as those above, and were removed at the time of the first reading for determination of the preformed lactic acid. In the last experiment it was found that the extent of this correction could be greatly reduced by suspending the tissue in warm oxygenated Ringer solution for 10 minutes before the experiment. Variations in the preformed lactic acid are the principal source of error. The figures given in Table V confirm, however, that the manometric findings accurately represent lactic acid formation.

Table V.

			Lactic acid					
			Chemical estimation			Manometric		
Exp. no.	Dry wt. of tissue mg.	Time mins.	Preformed mg.	Total mg.	Total *minus* preformed	CO_2 mm.³	Lactic acid mg.	Chemical / Manometric %
Rat-yolk-sac								
3	3·7	0	0·16	—	—	—	—	—
3	2·7	180	0·11	0·81	*0·70*	195	*0·78*	90
3	3·3	180	0·14	0·95	*0·81*	228	*0·91*	89
7	3·8	0	0·14	—	—	—	—	—
7	4·3	140	0·16	0·89	*0·73*	169	*0·68*	107
39	3·2	0	0·10	—	—	—	—	—
39	5·1	0	0·18	—	—	—	—	—
39	2·3	270	0·07	0·35	*0·28*	77	*0·31*	90
39	3·6	270	0·12	0·62	*0·50*	133·5	*0·53*	94
Cat-amnion								
41	3·9	0	0·04	—	—	—	—	—
41	3·8	0	0·02	—	—	—	—	—
41	6·2	0	0·08	—	—	—	—	—
41	4·5	240	0·04	0·67	*0·63*	158	*0·63*	100
41	5·1	240	0·04	0·90	*0·86*	228	*0·91*	95

Survey of results.

Nature of the medium. It is difficult to say what medium most nearly approaches the physiological for these membranes. In the living animal the membranes may enter into relationship with foetal blood, with the foetal

fluids, or with both of these, whilst both maternal and foetal blood enter the placenta. This question is of importance since the glycolysis is to some extent dependent on the nature of the medium used. Our glucolysis values in Ringer solution for rat outer membrane fall between those obtained by Negelein [1925] in inactivated horse-serum and those of Kumanomido [1928] in rat-serum. Indeed, the latter considers that serum exerts an inhibiting action on the glucolysis of embryonic tissues. Our experiments have therefore been carried out entirely in Ringer solution.

It should be emphasised that the above results were all obtained from experiments in 0·2 % solutions of the sugars. The arbitrary concentration of 0·2 % was adopted for glucose by Warburg; this concentration does not depart widely from the physiological level, while giving a sufficient sugar reserve, and for tumour tissue there is little fall in glucolysis when the concentration is decreased from 0·2 % to 0·1 % [Warburg, Posener and Negelein, 1924]. It should be noted, however, that 0·2 % is not necessarily the optimum concentration for tissues other than tumours or for sugars other than glucose. For our experiments such a concentration may not even be physiological, since considerably higher concentrations of fructose have been reported in the foetal fluids. It is, therefore, realised that a somewhat different picture might be obtained with other fructose concentrations. Again, the experiments were carried out at the p_H of blood, from which the p_H of the various foetal fluids may differ widely [Needham, 1931, p. 1543]. However, our use of the blood-p_H and of 0·2 % sugar concentration enables our measurements in glucose to be compared directly with those of other authors, whilst the fructolysis figures allow a comparison to be made of the attack on glucose and fructose under identical conditions.

Relative fructolysis. Since the autoglycolysis is often fairly large in embryonic tissues, direct comparison of the glucolysis and fructolysis does not always give a true indication of the relative extent to which the added sugars are attacked. When sugar is present in the medium the glycolysis is not always equal to the sum of the autoglycolysis and the lactic acid formed from the added sugar. This is shown by the fact that when in the later stages of an experiment the autoglycolysis falls to a low value, glycolysis in the sugar solution frequently does not fall by the same amount and may even remain constant at its initial rate throughout (see the Tables). We can only say that a sugar is attacked, when its addition causes the autoglycolysis to be exceeded, but for the reasons given above, the excess of glycolysis over autoglycolysis does not accurately represent the amount of the added sugar converted to lactic acid. This error, however, decreases with the autoglycolysis, so that we can best estimate the lactic acid formation from added sugar in the later period of the experiments when the autoglycolysis has fallen to a low value. The relative fructolysis is then described by the percentage

$$\frac{\text{Fructolysis} - \text{Autoglycolysis}}{\text{Glucolysis} - \text{Autoglycolysis}} \times 100.$$

The values of the relative fructolysis for the second hour of the various experiments are set out in the last columns of Tables I–IV, and are represented diagrammatically for the different tissues in Figs. 1 and 2, where the

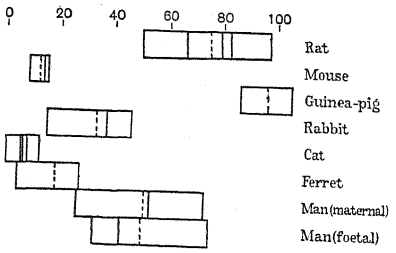


Fig. 1. Relative fructolysis of outer membrane.

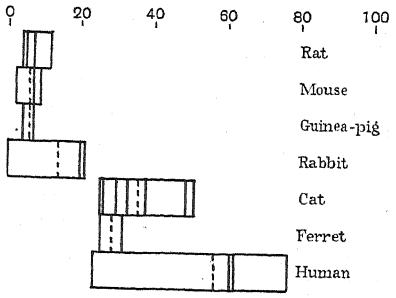


Fig. 2. Relative fructolysis of placenta.

values and their means are represented by continuous and dotted vertical lines respectively. Duplicates on the same tissue are shown as one. In a few experiments where observations over the second hour were not available, those of the second half-hour have been taken.

It will be seen that in some cases where the relative fructolysis is considerable the individual values are widely spread. Since the figures are obtained from tissues at different stages of pregnancy it might be supposed that the relative fructolysis is related to the age of the embryo. This, however, is not the case, for although the fructolysis falls with the glucolysis as pregnancy advances, the relative fructolysis shows no systematic variation. The spread of the relative fructolysis is not surprising with mixed tissues such as human and cat-placenta, but it is remarkable when it occurs in an apparently homogeneous membrane such as the rat-yolk-sac. However, even this latter membrane is by no means simple, since in the later stages it becomes villous [Robinson, 1892]. In view of the complexity of the tissues and the diverging results, it is impossible from our limited number of observations to make any far-reaching generalisations, but we wish to point out one or two inferences which are rendered probable by our findings.

Outer membrane. Considering first the yolk-sac of the rodents, it appears that the various species differ dramatically in their relative attack on fructose, rat and guinea-pig having high, and rabbit and mouse low, relative fructolysis. In contrast with this, the morphological appearances are almost identical, so much so that Grosser [1927] states: "Die Embryonalhüllen dagegen besitzen bei allen Nagern so viele auffällige gemeinsame, nur bei ihnen vorkommende Züge, dass die ganze Ordnung auf Grund der Hüllen ebensogut hätte aufgestellt werden können wie auf Grund des Zahnbaues."

Nevertheless, the previous history of the rabbit membranes is very different from that of the other three, and some difference in behaviour might be understood on this account; but the entirely different behaviour of rat and

mouse towards fructose is most surprising in view of their absolute similarity of development.

The allanto-chorion of Carnivora (cat and ferret) seems to attack fructose but little.

Placenta. In the later stages of pregnancy in the guinea-pig, rat and mouse the discoidal placenta is attached to the decidua over only a small portion of its surface. The conditions are therefore favourable for obtaining homogeneous material and consistent results. Such results indeed were obtained, the relative fructolysis being consistently low. The form of the placenta is rather different in the rabbit, and the relative fructolysis is possibly higher. It appears to be certain that the relative fructolysis is higher in the placenta of the cat and ferret than in that of the guinea-pig, rat and mouse. It is also seen from Exps. 40 and 43 (Table III) that the relative fructolysis is constant through the whole depth of the placenta, if we neglect the mid portion in Exp. 40 where the glucolysis is anomalously low.

The attack of the placenta on fructose does not depend on the extent of the erosion of the maternal uterine tissues effected by the trophoblast, for the haemochorial human placenta resembles in its relative fructolysis the syndesmochorial placenta of the carnivores rather than the haemochorial placenta of the rodents. We cannot therefore refer the fructolysis of the placenta of the carnivores to the retained maternal elements, nor can we say that the trophoblast itself behaves similarly in the placentae of the different species.

DISCUSSION.

"The fructose question," says Needham [1931, p. 1054], "is the most enigmatic aspect of embryonic carbohydrate metabolism." Whilst the appearance of fructose in pregnancy has been recorded, no way in which it may be utilised has so far been suggested. Its source is unknown, and in any case its presence in the amniotic and allantoic fluids cannot be explained until the vexed question of their origin has been settled. But the appearance of this sugar in pregnancy led us to look for a fructolysis of the foetal membranes and placenta, and this we have actually found to occur. At least one of the three tissues, outer membrane, amnion, or placenta, of the pregnant animal of every species examined (with the possible exception of the mouse in which the amnion was not investigated) was found to convert fructose into lactic acid. It would not be possible to correlate our findings with the occurrence of this sugar, which varies greatly in the different species, until complete data were available giving the amounts of fructose and glucose in the foetal fluids at different stages of pregnancy for the various species examined. The information which we have is slight, for only now are reliable methods for the determination of small quantities of fructose being developed. However, if in spite of this we examine our metabolic findings from the physiological standpoint, they are found to present several surprises. Paton, Watson and Kerr [1907]

found that among the animals we have used, fructose is a constant constituent of the allantoic and amniotic liquids of the cat and of the mixed fluids of rabbit, ferret and guinea-pig. Information on rat and mouse is lacking. We may note that in all four animals in which fructose is observed during gestation the amnion definitely fructolyses. This membrane has therefore to some extent adapted itself to the fructose-containing *milieu* which surrounds it. On the other hand, the allanto-chorion of the cat does not fructolyse, although the allantoic face of it is bathed in a medium which definitely contains fructose. Further, the placenta of the cat attacks fructose, so that chorion and placenta behave differently although they are both of trophoblastic origin.

We have shown that the whole embryo half-way through pregnancy does not utilise fructose for the formation of lactic acid, and that no special fructolytic power is developed in embryonic liver, lung or brain. Although they glucolyse readily, these tissues do not adapt themselves to the presence of the fructose in the circulating blood. It is clear also that the power to convert fructose into lactic acid is not a property always possessed by rapidly growing tissues. We have therefore neither shown a universal adaptation of embryonic tissues to the presence of fructose nor adduced a reason for its appearance during gestation. It may be that fructose is a by-product in embryonic metabolic processes of whose occurrence we are at present quite ignorant. It may be said that the fructose mystery deepens.

In seeking an explanation of the great variation in the relative attack on fructose and glucose shown by different tissues the following two main hypotheses might be advanced.

(1) Difference due to a different permeability of the tissues for fructose and glucose.

(2) Variation in the action of the "glycolytic enzyme"; or more exactly, of the system of enzymes and co-enzymes responsible for the conversion of added hexose into lactic acid. This might be due either to different modes of attack in the various tissues (different or partially different enzyme systems) or to a different reactivity towards different forms of the sugars, so that with one tissue one form (*e.g.* *h*-fructose) might have a higher affinity for the enzyme system than the other (*e.g.* *n*-fructose), whilst in another tissue the converse might hold (nomenclature of Schlubach [1932]).

Similar problems in the selective fermentation of sugar solutions by yeast have been much investigated [Willstätter and Sobotka, 1922; Hopkins, 1931; Fernbach, Schoen and Mori, 1927; Dawson, 1932]. Here most workers do not favour the permeability theory, though direct evidence is difficult to obtain. In animal tissues observations on the relative permeability of rat intestinal canal to fructose and glucose have been made by Cori [1925] who found that this tissue was freely permeable to fructose, the ratio of permeabilities of glucose and fructose being approximately 2 : 1.

It appears unlikely that a different permeability is the main factor in

deciding the extent of attack on fructose and glucose by the tissues described in the present paper. If this were so it would be necessary to assume that several morphologically and embryologically almost identical membranes (such for example as the yolk-sac of rat and mouse) which show a high and almost equal rate of attack on glucose, differ so widely in their permeability to fructose that the relative fructolysis is caused thereby to differ from nearly 0 to 100 % of the glucolysis. It is true that this possibility has not been excluded by direct experiment, and that this might be possible by a study of the oxidative reactions, as was done by Loebel [1925] for brain tissue. If those tissues which do not fructolyse should be found to oxidise fructose this would be a definite proof of its diffusion into the cells. Experiments on this point are at present in hand but some difficulty is caused since we find that the respiration of these embryonic tissues, unlike that of brain, often does not fall off markedly in the absence of added substrate, and the clear demonstration that fructose is being oxidised is thus rendered more difficult.

It should be remarked that the possibility that permeability regulates the attack on the sugar has not as yet been definitely excluded even for glucose as substrate. It has yet to be established how far the gradation in the ability of the various tissues to glucolyse [Warburg, 1927], which coincides with that in their power to oxidise, carbohydrate [Dickens and Šimer, 1930], is to be attributed to enzymic rather than diffusion differences. Nevertheless, it is usual to attribute the differing ability of various types of tissue to convert glucose into lactic acid, or to oxidise it, not to their differing permeabilities to the sugar but to the different activities of the respective enzyme systems in them. It is therefore at least as justifiable to exclude permeability questions as the deciding factor in the present problem.

It is noteworthy that in the normal tissues previously observed to convert fructose into lactic acid (muscle, liver) several apparent differences from the other body tissues are evident. These are the only tissues in which resynthesis through the Meyerhof cycle has been experimentally demonstrated. Muscle tissue attacks preferentially polysaccharides such as glycogen, starch and trihexosan, whilst the simple hexoses are less affected. The other tissues, on the other hand, react, often vigorously, with glucose and mannose converting these sugars into lactic acid, whilst fructose is hardly attacked by them. Loebel [1925] found that brain tissue could not form lactic acid from fructose though it oxidised this sugar as readily as glucose or lactic acid, thus showing that it is not impermeable to fructose. According to Ashford and Holmes [1929] the lactic acid formation in brain is of two types, the one without and the other with preliminary interaction of the carbohydrate with phosphoric acid; of these the former greatly predominates, whilst the latter, which involves glycogen, accounts for only a small fraction of the total anaerobic glycolysis. Thus in brain inability to fructolyse is associated with the absence of phosphorylation. On the other hand, Meyerhof [1930, p. 155] has shown that fructose is broken down to lactic acid rapidly by his glycolytic muscle-

extract, provided that hexokinase be added to the system. During the initial phosphorylation period the attack on fructose was twice as rapid as that on glucose. In view of these observations, Meyerhof [1930, p. 194] has suggested that the special suitability of fructose for lactic acid formation in muscle and muscle-extract is analogous to the behaviour of these two sugars in alcoholic fermentation and can be attributed to the fact that fructose is more readily phosphorylated than is glucose. "Mann kann vielleicht das abweichende Verhalten der übrigen Gewebe darauf beziehen, dass bei der Zuckerspaltung hier intermediär keine Phosphorsäureester gebildet werden. Denn ebensowenig wie Glykogen wird die Hexosephosphorsäure von diesen Geweben gespalten, mit alleiniger Ausnahme der Leber, wo ja auch Glykogen abgebaut wird und der Spaltungsmechanismus mit dem des Muskels näher übereinstimmt." To this example (liver) we may probably add a number of embryonic tissues which are also able to break down fructose to lactic acid, but possess the advantage of attacking both glucose and fructose strongly. It is probable that these tissues, showing every degree of fructolysis from nearly 0 to 100 % of the glucolysis, will provide a peculiarly suitable material for the investigation of the different mechanisms by which carbohydrate is attacked by animal tissues.

At the present time we would, therefore, merely indicate the nature of the problem. The embryonic tissues described in this paper appear to resemble muscle and liver in little but that some of them may contain fairly high concentrations of glycogen [Grosser, 1927, pp. 86, 391]. However, if Meyerhof's explanation should prove to be applicable to the different behaviour of glucose and fructose in our embryonic tissues, we should expect to find the two types of attack—with and without preliminary phosphorylation of the sugar—occurring to different extents in tissues whose behaviour towards glucose apparently gives no indication of any difference in metabolism. A striking example of this would be given by the yolk-sac of the rat and mouse, the former showing purely phosphorylation and the latter none. On the other hand, the two mechanisms might be combined in any one tissue in any proportion, resulting in an attack on fructose intermediate between the autoglycolysis and the glucolysis. This fundamental question of carbohydrate utilisation by animal tissues, which for lack of suitable material has not yet received any systematic investigation, obviously cannot be decided on the evidence at present available. In view of its importance to carbohydrate metabolism in general, we propose to continue investigations on the suitable tissues provided by the embryonic material whose behaviour has been described in the present paper.

SUMMARY.

1. A study has been made of the anaerobic conversion of fructose and glucose into lactic acid by the foetal membranes (outer membrane, amnion, yolk-sac, allantois), the placental tissues, the whole embryo and the tissues

of the later embryo (liver, lung, brain and skin). The measurements were made throughout at p_H 7·5 in bicarbonate-Ringer solution containing 0·2 % of the sugar.

2. Tissues of the rat, mouse, guinea-pig, rabbit, cat and ferret at different stages of pregnancy were examined. Whilst glucose is converted into lactic acid with great readiness by most embryonic tissues, the attack on fructose depends on both the tissue and the species, varying from zero to a value equal to that observed with the same tissue in glucose.

3. For a given tissue the figures for the relative attack on fructose and glucose may show a considerable spread. In view of this some of the results cannot be adequately summarised, but the following are the most probable conclusions.

Tissues with attack on fructose approaching or equalling that on glucose. Outer membrane of rat and guinea-pig; allantois of rabbit.

Tissues with intermediate attack on fructose. Placenta of ferret and cat; yolk-sac of cat.

Tissues with little attack on fructose. Placenta of rabbit, guinea-pig, rat and mouse; outer membrane of mouse, cat and ferret. Tissues of the embryo.

4. The amnion attacks fructose, though to a different degree in different species.

5. The placenta and peripheral membrane from the full-time human after-birth both attack fructose, though to a less extent than glucose.

6. Neither the whole embryo at mid-term (rat, rabbit) nor the isolated tissues of the later embryo mentioned under 1 above, are able to convert fructose into lactic acid, although fructose is known to be present in the foetal blood.

7. It is concluded that in all species in which fructose is known to be present in the foetal fluids, it is a possible source of energy to at least one of the extra-embryonic foetal tissues.

8. There is no apparent correlation between the fructolytic power and the development and morphology of the tissues examined.

9. In view of Meyerhof's suggestion that ability or inability to fructolyse is connected with the existence or absence of preliminary phosphorylation, the tissues examined may well prove of value in the elucidation of the early stages of intermediate carbohydrate metabolism.

We wish to express our thanks to Prof. E. C. Dodds for his interest in these experiments, and to Dr J. H. Woodger and Dr L. W. Proger of this Hospital for their advice, and for histological examination of some of our material.

One of us (F. D.) is a member of the Scientific Staff of the Medical Research Council, and the other (G. D. G.) wishes to make acknowledgments to the Halley Stewart Trust Fund for a scholarship.

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CL. THE PREPARATION OF METHIONINE FROM CASEINOGEN.

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In the course of some work on the unidentified sulphur compounds in proteins I have found that the methods that have been used for the isolation of methionine from a protein hydrolysate are very unsatisfactory. All the methods that have been described, except that of Odake [1925] who isolated 0·0001 % of methionine from yeast, are simply modifications of Mueller's [1923] original method and involve the precipitation of the mercury compound of methionine mixed with a large amount of basic mercuric sulphate. The following yields have been obtained from caseinogen. Mueller [1923] 0·2–0·4 %, Barger and Coyne [1928] 0·1–0·3 %, du Vigneaud and Meyer [1931] 0·1–0·2 %. These low yields, and the laborious nature of the isolation, justified Barger and Weichselbaum [1931] in concluding that it was easier to make this amino-acid synthetically.

In this paper a method of preparation is described which is very much simpler than those that have already been described and which gives better yields; 1·0 % of methionine is readily obtained from caseinogen and, by working up the final mother-liquor the yield may be raised to 1·4 %.

It became obvious during the preliminary work that the precipitation of methionine as its mercury compound was unsatisfactory in the presence of large amounts of other amino-acids. An initial partial separation of the hydrolysate is therefore imperative and is most easily carried out by means of butyl alcohol. In strongly acid solution methionine is appreciably soluble in the alcohol, it may therefore be concentrated by the ordinary Dakin [1918] procedure. I have found it more convenient, however, to extract the methionine from the butyl alcohol with sodium hydroxide instead of distilling off the alcohol from the amino-acid mixture. I have carried out this double extraction in a separating funnel but it would be easier on a large scale to do it continuously. The hydrolysate should be purified somewhat, before extracting, by precipitation with lead acetate; if this precipitation is omitted a semi-solid foam is obtained when the hydrolysate is shaken with butyl alcohol. Picric acid is equally efficacious if for any reason the presence of lead in the extracted fluid is to be avoided. It does, however, precipitate a tar from the hydrolysate and for this reason I prefer lead.

Experimental.

200 g. caseinogen are hydrolysed by boiling for 14 hours with 200 cc. of water and 400 cc. of concentrated hydrochloric acid. The hydrolysate is distilled *in vacuo* till it becomes very viscous; the residue is then dissolved in 500 cc. of water. It dissolves slowly and it is generally necessary to heat the mixture. When it has cooled, 200 cc. of saturated normal lead acetate are added and the precipitate of lead chloride and tarry material is removed by filtration.

A sample of the filtrate is titrated with sodium hydroxide using bromophenol blue as an external indicator. It should be equivalent to 1–1·5 times its volume of *N* NaOH. This strongly acid solution is now extracted with 2 litres of technical butyl alcohol in a large separating funnel and the aqueous layer is run off. Sometimes it is difficult to see the interface since each layer is dark brown in colour; this difficulty can be overcome by having the apparatus in a poorly lit room and holding a powerful electric lamp close to it. The butyl alcohol in the funnel is now shaken with sufficient 10 % sodium hydroxide solution (about 250 cc.) to make it neutral. A large excess of alkali should be avoided, but it makes little difference whether the mixture is left neutral or is made definitely alkaline. The aqueous layer is separated after being left for an hour or so to settle thoroughly. It is important that the sediment which collects at the interface during the first (acid) extraction should be removed with the aqueous layer whereas that which collects during the second (alkaline) extraction should be left in the separating funnel. The acid aqueous solution from the first extraction is now returned to the funnel and extracted again with the same butyl alcohol and the butyl alcohol re-extracted with 5 % sodium hydroxide (about 300 cc. will probably be required). This alkaline aqueous extract is mixed with the first lot.

After two more double extractions as described above the greater part of the methionine, along with other amino-acids, is obtained in the alkaline solution. The same amount of butyl alcohol has been used successfully to extract as much as 800 g. of hydrolysed caseinogen, in this case the double extraction was repeated 12 times. After every second extraction 30 cc. of conc. hydrochloric acid should be added to the hydrolysate. After the last alkaline extraction a further extraction with 200 cc. of water will bring out a little more methionine.

The combined alkaline extracts are now distilled *in vacuo* till all the dissolved butyl alcohol has been removed and the volume is about 500 cc. When cold the solution is filtered and the solid is boiled with about 200 cc. of water, cooled and filtered again. The combined filtrates are brought to p_H 5 with HCl, and 200 g. of mercuric acetate dissolved in about 500 cc. of 3 % acetic acid are added. The volume should now be 1000–1500 cc.; if it is smaller than this the precipitation will be incomplete. Precipitation is complete in a minute or two and the mixture may be filtered immediately. The solid is sucked dry on the

filter, immediately ground up with 400–500 cc. of water and again filtered. The mercury precipitate should never be allowed to become parched or the subsequent extraction with baryta will be unsatisfactory. The precipitate is suspended in water and added to 600 cc. of boiling $N/3$ barium hydroxide. The mixture is heated to boiling, solid barium hydroxide being added if necessary to make the solution strongly alkaline to thymolphthalein. The mixture is filtered hot and the solid is extracted again in the same way with 700 cc. of $N/7$ barium hydroxide.

Barium is removed fairly exactly from the combined filtrates with sulphuric acid. The amount of sulphuric acid finally present should not be greater than 1 cc. of $2N$. The mixture is filtered and the filtrate distilled *in vacuo* to dryness to remove as much as possible of the acetic and hydrochloric acids. The residue is dissolved in 30 cc. of water and poured into a boiling solution of 60 g. $HgCl_2$ in 120 cc. of water. The mixture is cooled in a freezing mixture till crystals of ice separate. It is then left for an hour or two at 0° and filtered. The putty-like mass of methionine-mercury compound is kneaded with ice-cold water; this is added to the filtrate. A further quantity of less pure methionine can be obtained by adding 6–10 g. of mercuric acetate, in the form of a 30 % solution, to this filtrate and working up the precipitate as if it were a mercuric chloride precipitate.

The mercury compound is suspended in 250 cc. of water and decomposed with H_2S; the decomposition is rather slow. The filtrate from mercuric sulphide is distilled *in vacuo* nearly to dryness. Care is required at this point, for if the distillation is not carried far enough a large amount of HCl will remain with the methionine. This will necessitate the addition of a large amount of pyridine in the next stage and the yield of methionine will be small because it is appreciably soluble in alcohol in the presence of pyridine hydrochloride. If on the other hand the distillation is too violent solid methionine hydrochloride[1] will blow down the condenser as a fine dust. I have found it safest to connect the distillation flask to the condenser by means of a piece of pressure tubing, this can be clamped off at the critical moment. 30 cc. of alcohol are used to dissolve the solid methionine hydrochloride; if necessary the alcoholic solution is filtered and the flask washed out with a further 15 cc. of alcohol.

Pyridine is now added until a drop of the solution on a tile is alkaline to bromophenyl blue; 2 cc. more of pyridine are added and the mixture cooled for some hours in a freezing mixture. It is filtered and the solid washed with a little alcohol.

Yield, 1·8 g. of methionine of 95 % purity.

When measured in 3 % aqueous solution $[\alpha]_{5461}^{21^\circ} = -7{\cdot}9°$.

From the mercuric acetate precipitate a further 0·8 g. is obtained. This is only 75 % pure but may be purified by reprecipitation with mercuric chloride.

[1] Methionine, like ethylcysteine, forms a hydrochloride, which crystallises from strong hydrochloric acid in clusters of long needles and does not lose hydrochloric acid when dried *in vacuo*. It melts sharply at 174° (uncorr.) but solidifies again, and on further heating melts at 210–223°. Found: N, 7·2; S, 17·22; Cl, 19·4. Calculated: N, 7·54; S, 17·25; Cl, 19·12 %.

In each case the alcoholic mother-liquor contains sulphur to the extent of about 1·5 mg./cc. In this case there is therefore about 150 mg. of sulphur still present in the mother-liquor. The greater part of this is in the form of methionine and I have isolated it in about 70 % yield (reckoned on the sulphur content) by making the combined mother-liquors from several preparations alkaline (with barium hydroxide) and distilling *in vacuo* to get rid of pyridine, followed by acidification (with sulphuric acid) and precipitation with mercuric chloride.

The yield of methionine isolated in this way is therefore 1·4 %. This is considerably less than the actual methionine content of caseinogen, for more may be obtained by more prolonged extraction with butyl alcohol and also by precipitating with a larger amount of mercuric acetate. Both of these procedures, however, involve more labour than the extra yield is worth.

Methionine may also be isolated by following the method already described but with the omission of the butyl alcohol extractions. In this case the yield is much smaller and is very variable; the method then differs from those that have already been described by other workers only in the shortening of some of the processes and gives a very similar yield, *i.e.* 0·25–0·7 %.

Discussion.

The commercial caseinogen which has been used in this work contains 0·6 % of sulphur (uncorrected for moisture or ash) and from it 1·4 % of methionine has been isolated; this, since methionine contains 21·5 % of sulphur, corresponds to 0·3 % of sulphur in the protein. It is clear therefore that methionine is the principal sulphur-containing constituent of caseinogen.

The yield of methionine is not quite high enough definitely to rule out the possibility of a third sulphur-containing amino-acid. Most workers have put the cystine content of caseinogen at 0·3 % [*e.g.* Sullivan, 1929]; on this basis there is a considerable amount of sulphur in caseinogen still unaccounted for but it is probable that part of this is methionine. Baernstein [1930], by oxidation with iodine, finds 0·94 % of cystine in caseinogen; on this basis there can be no other sulphur-containing constituent. Sherman and Woods [1925], by feeding experiments, found a still higher value, 1·2–2·5 %. This value is clearly impossible and it seems from the recent work of Jackson and Block [1931] and of Weichselbaum, Weichselbaum and Stewart [1932] that their method of estimation does not distinguish between methionine and cystine. Steudel and Schumann [1929] found, by the Folin-Looney [1922] technique, 2·6 % of "cystine" in caseinogen which had been treated with nitrous acid. This value, like that of Sherman and Woods, appears to be the sum of the cystine and the methionine contents of the protein, but the nature of the reaction involved is obscure; free methionine, on treatment with nitrous acid, does not give a product that reacts with the Folin-Looney reagent.

SUMMARY.

The preparation of methionine from the butyl alcohol extract of an acid hydrolysate of caseinogen is described.

The yield is 1·4 %.

I am deeply indebted to Sir F. G. Hopkins for his interest in this work.

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CLI. THE DETERMINATION OF CELLULOSE IN SOIL[1].

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Introductory.

During the course of an investigation into the decomposition of green manures in soil it was necessary to determine the amount of cellulose that underwent changes at the different stages of fermentation. Charpentier [1920] has shown that when cellulose, in the form of filter-paper, is mixed with soil it can be recovered quantitatively by extraction with Schweitzer's reagent, an observation later confirmed by Barthel and Bengtsson [1924]. This method, however, cannot be applied directly to soils mixed with plant materials, as the plant tissues contain, amongst other constituents, hemicelluloses and lignin which interfere with the extraction of cellulose with Schweitzer's reagent. The hemicelluloses are to a certain extent soluble in Schweitzer's reagent and are precipitated with alcohol. Lignin on the other hand acts as a barrier to the complete dissolution of cellulose in Schweitzer's reagent.

Waksman and Tenney [1927] suggested treating the material with a 5 % solution of NaOH for 30 minutes at 15 lbs. pressure in order to remove the hemicelluloses and lignin before extracting the cellulose with Schweitzer's reagent. Solutions of sodium hydroxide of strength greater than 1–2 % are known to attack cellulose, so that any treatment in which the plant material is exposed to the action of alkali of more than 1–2 % strength at high temperatures is bound to give a low yield of cellulose. Bengtsson [1924] proposed treating the soil mixed with plant materials with a solution of sodium bisulphite in hydrochloric acid at a temperature of 100° for 72 hours and then extracting cellulose with Schweitzer's reagent. Apart from the fact that the sulphite process of separating cellulose gives lower yields than the Cross and Bevan method, the period of digestion required for the preliminary treatment of the material makes the process too long.

With a view to the elimination of these disadvantages an attempt was made to find a suitable method for the estimation of cellulose in plant materials mixed with soil.

[1] This paper is an abridged form of part of a thesis approved for the degree of Ph.D. in the University of London.

Experimental.

Some preliminary experiments were carried out on a sample of oat straw whose cellulose content was known. It contained 39·3 % cellulose by Jenkins's method [1930] which gives as good results as the Cross and Bevan method. In all subsequent experiments this figure was taken as the standard with which the results of the various methods tried were compared.

An attempt was first made to see if Schweitzer's reagent could extract all the cellulose from the straw after the hemicelluloses were removed as suggested by Waksman [1927]. One g. portions of straw were treated with 100 cc. of 1 % NaOH and 1 % H_2SO_4 at boiling temperature for half an hour with each reagent. The residue, after thorough washing and drying, was shaken with 100 cc. of Schweitzer's reagent for one hour[1] and the cellulose estimated according to Charpentier's process. The residue was re-extracted twice with Schweitzer's reagent and cellulose precipitated each time as before. The results (Table I) show that even by extracting the material three times, a part of the

Table I.

	1st Extraction	2nd Extraction	3rd Extraction	Total
Cellulose on dry matter, %	29·5 27·6	2·94 3·05	2·29 1·66	34·37 32·31

cellulose only could be recovered. There is a progressive dissolution of cellulose, its complete dissolution being hindered by lignin with which it is supposed to be encrusted.

The use of chlorine gas for removing lignin, apart from its other drawbacks, is ruled out where the material under treatment is mixed with soil. The sulphite process of Bengtsson, already referred to, was tried on one g. portions of the oat straw (Table II).

Table II.

	1st Extraction	2nd Extraction	Total
Cellulose % on dry matter	36·7 37·5	0·86 0·50	37·56 38·00

Though the removal of lignin has increased the amount of cellulose that could be extracted with Schweitzer's reagent, yet the yield is not up to the standard. This suggests that a part of the cellulose has been attacked during the preliminary digestion with the bisulphite. It is clear that it is necessary to remove lignin from the plant tissues before the cellulose can be completely dissolved by the Schweitzer's reagent, and yet the reagent employed must be such as to have a minimum effect on the cellulose. Jenkins's method of chlorinating lignin by means of sodium hypochlorite in an alkaline solution suggested itself and was next tried. One g. portions of the straw were subjected to the

[1] In some preliminary experiments it was found that shaking for one hour extracted as much cellulose as shaking for 6 or 24 hours.

dilute alkali and acid treatments as before and the residues therefrom were treated twice with sodium hypochlorite as described by Jenkins. The excess of hypochlorite was removed and the residues, after drying, were extracted with 100 cc. of Schweitzer's reagent and cellulose was estimated as before (Table III).

Table III.

No. of determination	Cellulose % on dry matter
1	40·15
2	39·96
3	40·05
4	39·07
5	40·82
Mean value	40·01 ± 0·28

The Schweitzer's reagent is now able to extract the whole of the cellulose at one extraction and the results agree well with the standard.

The straw was now mixed with a light sandy soil, and the mixture subjected to the same treatments as with straw alone. 10 g. of the soil mixed with 1 g. of the straw were used for analysis (Table IV).

Table IV.

		Cellulose in mixture of soil + straw	
No. of determination	Cellulose in soil %	Amount obtained	Amount calculated
1	0·0627	0·3955	
2	0·0546	0·3987	
3	0·0627	0·3915	
4	—	0·3858	
5	—	0·3851	
Mean value	0·0600 ± 0·0027	0·3913 ± 0·0027	0·4060

The results show that almost the whole of the cellulose added to the soil in the form of straw can be recovered by the method of analysis employed. A part of the cellulose, 0·0147 g. could not be recovered from the mixture. This loss of cellulose which was also observed by Charpentier and others is attributed by them to adsorption by the soil. Whatever the explanation be, it seems certain that a part of the added cellulose, in whatever form it is added, is always retained by the soil and is not recovered by the method of analysis used.

The method was then applied to different plant materials and to mixtures of these plant materials with soil. In all cases 10 g. of the soil together with 1 g. of the plant material were taken for analysis. Table V shows the results of analysis of plant materials alone. In Table VI the figures are given for the same plant materials mixed with the soil.

The results show that the cellulose added to the soil in the form of plant materials of widely different nature and age can be recovered almost quantitatively.

Table V.

No. of determinations	Cellulose on 100 g. dry matter: Young tares	Young tares by Jenkins's method	Young mustard	Sugar beet tops	Sugar beet tops by Jenkins's method	Mature mustard
1	9·83	9·62	9·42	7·18	7·87	22·90
2	10·48	10·11	8·60	8·12	7·79	23·75
3	9·21	—	—	8·09	—	—
4	9·52	—	—	7·61	—	—
5	9·83	—	—	6·53	—	—
6	9·28	—	—	6·79	—	—
Mean value	9·69	9·87	9·01	7·39	7·83	23·32
Standard error	0·18	—	0·51	0·26	—	0·42

Table VI.

	Cellulose in 10 g. soil + 1 g. plant material, expressed on dry matter: Cellulose g.	Average value	Amount calculated	Difference
Young tares + soil	0·0926 0·1015	0·0970	0·1028	0·0058
Young mustard + soil	0·0968 0·0833	0·0901	0·0960	0·0059
Sugar beet tops + soil	0·0731 0·0731 0·0745 0·0731	0·0735	0·0798	0·0063
Mature mustard + soil	0·2375 0·2350	0·2362	0·2391	0·0029

Description of method.

Ten g. of soil are heated in a beaker with 100 cc. of 1 % NaOH and allowed to boil for 20–30 minutes, maintaining a constant volume during heating. The soil is allowed to settle; this may be hastened by making the suspension slightly acid. The upper liquid is filtered off and the residue washed twice with hot water. The material on the filter is transferred to the beaker and the soil heated with 100 cc. of 1 % hydrochloric acid for about 20 minutes, maintaining a constant volume of liquid. After settling, the upper liquid is filtered off and the residue washed free of acid with hot water. The material on the filter-paper is returned to the beaker, 5 cc. of sodium hypochlorite, having 15 % available chlorine, are added and the volume is made up to 100 cc. The liquid must remain alkaline. It is allowed to stand for half an hour in the cold with three or four shakings during the interval. The upper liquid is filtered and the material on the filter returned to the beaker, another 5 cc. of the sodium hypochlorite solution added and the solution made up to 100 cc. and allowed to stand for half an hour with shakings as before. As much as possible of the upper liquid is filtered and to the residue in the beaker a dilute solution of H_2O_2 (10 cc. of 20 vol. H_2O_2 made up to 100 cc.) is gently added till effervescence

ceases, excess being avoided. The suspension is allowed to settle, being hastened with HCl if necessary, and the clear liquid is filtered through the same filter-paper. The residue in the beaker is transferred to an evaporating dish and washed thoroughly free of acid with hot water by decantation. The material on the filter-paper is transferred to the dish and the residue evaporated to dryness on a water-bath.

When the residue is completely dry, it is transferred to a suitable bottle for extraction with Schweitzer's reagent. 100 cc. of Schweitzer's reagent are added and the material shaken for one hour. It is then allowed to settle, preferably overnight. More than 50 cc. are filtered, a suction pump being used if necessary. To 50 cc. of the filtrate in a 400 cc. beaker 200 cc. of 80 % alcohol are added, and the precipitate is allowed to settle overnight. It is then filtered by decantation through a suitable filter aided by suction, Jena glass filter-crucible No. 1G4 being very convenient. After the upper liquid has all been filtered, 50 cc. of a mixture of alcohol and HCl (40 cc. of 80 % alcohol *plus* 10 cc. HCl) are added to the precipitate; the mixture is shaken till all the copper hydroxide is dissolved and is then allowed to stand for about an hour. The upper liquid is filtered through the same filter, the precipitate washed free of copper with hot water, and transferred to the crucible, washed further with hot water, then with alcohol and ether in turn as directed by Charpentier. The crucible is dried in an oven for about one hour; the cellulose is transferred to a weighed crucible, weighed, ignited and again weighed. The loss of weight on ignition multiplied by 2 represents the amount of cellulose in 10 g. of the soil.

Summary.

1. A method is described for the determination of cellulose in soil mixed with plant materials. It is essentially a combination of the Jenkins and Charpentier methods. The soil is treated with hot dilute alkali and acid and then with a solution of sodium hypochlorite in the cold. Cellulose is then extracted from the residue with Schweitzer's reagent, precipitated with alcohol and determined by loss of weight on ignition.

2. The results obtained by this method on plant materials alone agree very closely with those given by the hypochlorite method of Jenkins.

3. It is shown that it is necessary to remove the lignin from the plant tissues, otherwise it is not possible to obtain a quantitative yield of cellulose by extraction with Schweitzer's reagent.

4. The method has proved its value in recovering almost the whole of the cellulose added, when different plant materials are mixed with soil. A part of the cellulose is, however, retained by the soil and is not extracted by the Schweitzer's reagent.

5. No attempt has been made to apply the method to different types of soil.

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CLII. OXIDATIONS CATALYSED BY HORSE-RADISH- AND MILK-PEROXIDASES.

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In a previous paper [Elliott, 1932] a method was described for discovering whether any substance was oxidisable by H_2O_2 under the influence of peroxidase catalysis, and it was shown that the range of substances whose oxidation was catalysed by milk-peroxidase was very limited. Very dilute H_2O_2 with the enzyme had no effect on the various fatty acids, fats, sugars, most amino-acids, and other possible metabolites tried. Other than those compounds used in ordinary tests for peroxidase, the only substances found to be oxidised were tryptophan and tyrosine, and the oxidation of nitrite, previously observed by others, was fully confirmed.

The peroxidase from horseradish is one of the most intensively studied of enzymes and yet very little has up to now been known of its activity as a catalyst of biological oxidations. This fact and the interest of comparing the activity of an enzyme of plant origin with the corresponding enzyme from an animal source, led me to apply to horseradish-peroxidase preparations the same method as utilised with the milk-enzyme. The results obtained indicated a few more experiments to be made with the milk-enzyme.

Preparation of horseradish-peroxidase.

For this work it was necessary to have an enzyme preparation free from reducing substances and catalase, but a highly concentrated enzyme was not required. The "crude" product of Willstätter and Stoll [1918] would probably have been suitable but its preparation is laborious and occupies several weeks. The following entirely different method was found to be very satisfactory.

1470 g. of horseradish were washed and without further treatment chopped up and passed through an ordinary meat-mincer. To the mince 2 litres[2] of distilled water were added and the mixture stirred frequently during 2 hours. The liquid was squeezed through a cloth, and another extraction was made with 2 litres of water in the same way. A third extraction was done with 2 litres allowing the mixture to stand overnight before straining off the liquid. Qualitative tests showed that the third extract was considerably less active

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[2] It has since been found better to make the 3 or 4 extractions with only one litre each time.

than the first two, and though a large amount of enzyme remained in the solid it was not economical to extract it further.

The combined extracts, 6400 cc. including juice, were centrifuged or filtered through fluted filter-papers, and the translucent liquid was then fully saturated with ammonium sulphate. This precipitated the enzyme completely, together with a considerable amount of insoluble impurity. The precipitate was separated by centrifuging or on Büchner funnels, and lixiviated with a little water (250 cc. in this example, but preferably less). The turbid solution was dialysed through animal (or parchment) membrane for 3 days with frequent changes of outside water, until salt-free. The fluid, which had doubled in volume during the dialysis, was filtered clear and twice its volume, 1000 cc., of denatured[1] alcohol was added. The precipitate which formed was collected by centrifuging and dried in a vacuum desiccator. To prevent the alcohol paste splashing during the drying, it was covered by a slanting large perforated disc.

470 mg. of dried and ground up powder were obtained, having a purpurogallin number of 25·3.

To the alcoholic liquid a further 2 volumes, 1000 cc., of alcohol was added and the precipitate collected and dried as before.

125 mg. of a powder of purpurogallin number 212 were obtained.

In another preparation carried out as above the whole four volumes of alcohol were added together and the product obtained weighed 410 mg. with purpurogallin number 95·2.

The material obtained by this method is not easily soluble, and for estimation and other purposes it is necessary to grind it up carefully to a paste with very little water before diluting.

The whole preparation, including the period of dialysis can easily be finished in one week.

Oxidations by peroxidase and H_2O_2.

The method used was that described in the previous paper (pp. 16 and 19), the only change being that the experiments were made at 20° instead of at 37°.

Nitrite. It was previously shown that nitrite is quantitatively oxidised by H_2O_2 under the influence of milk-peroxidase catalysis; this oxidation was used as a test of the method, and hence nitrite was the first substance tried with the plant-enzyme. The remarkable result was obtained that horseradish-peroxidase is completely unable to catalyse the oxidation of nitrite.

Experiments carried out exactly as with milk-peroxidase showed no H_2O_2 usage at all, and, using very small amounts of nitrite, the Griess-Ilosvay test at the end of experiments showed no diminution in nitrite whatever. Experiments were made with the same result at room temperature and at 37°, at various hydrogen ion concentrations between p_H 5·7 and 7, using more H_2O_2,

[1] EtOH 96 %, MeOH 2·5 %.

or increasing the time allowed for the reaction, and with various preparations of the enzyme including a Willstätter adsorption product kindly given me by Dr H. Sutter.

The following is the summary of one experiment.

Flasks A and B each contained 20 cc. of a solution of 55 mg. of an enzyme preparation of P.Z.[1] 54, in phosphate buffer at p_H 6. A, contained 3 cc. of 3 mg./cc. sodium nitrite solution; B, 3 cc. of water. During 45 minutes at 20°, 10 cc. of 0·02 % H_2O_2 were run into each flask; after a further 15 minutes small samples tested with *p*-phenylenediamine showed by immediate coloration that both active enzyme and H_2O_2 were still present. On estimating the H_2O_2 remaining by the MnO_2 method in neutral solution, 0·64 cc. and 0·68 cc. of O_2 were liberated from the contents of flasks A and B respectively. No usage of H_2O_2 had therefore taken place. A similar experiment using only 0·5 cc. of nitrite solution, the H_2O_2 therefore being in excess, showed the same intensity of colour with the Griess-Ilosvay reagent after the experiment as a standard solution of the nitrite of the same dilution.

Qualitative tests showed that the enzyme was still active at the end of the experiments. In order to make sure that no inactivation at all was caused by the nitrous acid at the slightly acid reaction, the following experiment was made. To 5 cc. of a solution of 5 mg. of peroxidase of P.Z. 54 in phosphate buffer p_H 5·5, 2 cc. of nitrite solution 3 mg./cc. were added and the mixture kept for 1½ hours. On diluting and carrying out an estimation, the enzyme was found to have lost none of its activity.

Thurlow [1925] was also unable to show the oxidation of nitrite by plant-peroxidase but believed this to be due to reducing substances in her crude preparation reacting with the nitrate formed. My method excludes this possibility since it shows that not only does nitrite not disappear, but H_2O_2 is not used up.

The slight colour mentioned in the last paper as developing when H_2O_2 was added to nitrite and milk-peroxidase solution was again observed with the plant-peroxidase. It is not formed in the absence of nitrite or of enzyme and is therefore difficult to account for unless traces of nitrite are oxidised and the trace of nascent nitrate formed gives a xanthoproteic reaction. The colour deepens with ammonia.

It is necessary here to correct an error in my previous paper. I have found that in acid medium nitrous acid reduces H_2O_2 quantitatively, so that H_2O_2 cannot be estimated in acidified solutions containing nitrite. In Tables III, IV, V and VI of that paper the fact that no O_2 was liberated by MnO_2 in acidified solution in the presence of excess of nitrite is therefore meaningless. But in all those tables the qualitative tests recorded show that the H_2O_2 had all been used up before adding acid. The estimations in Table II showing the oxidation of nitrite were done in neutral solution, as also were the experiments on nitrite recorded here above. These estimations and all results and conclusions are therefore perfectly valid. Careful controls have shown that nitrite in neutral solution and in buffer as acid as p_H 5·5, does not affect the H_2O_2 estimation by MnO_2.

[1] P.Z. (=Purpurogallin Zahl) is the usual abbreviation for Purpurogallin Number as defined by Willstätter and Stoll [1918].

Possible metabolites. Besides nitrite the two other substances found to be oxidised under the influence of milk-peroxidase catalysis were tryptophan and tyrosine. With the plant-enzyme tyrosine was found to be oxidised, but no sign of oxidation of tryptophan could be observed; we have here therefore another remarkable difference between the two enzymes. The following compounds of different types were tried with the plant-peroxidase preparation and found, as with milk-peroxidase, not to be oxidisable; *viz.* formate, acetate, lactate, oleate, glucose, fructose, glycerol, ethyl alcohol, acetaldehyde, glycine, glutamic acid, phenylalanine, histidine, brucine.

It was previously shown that dihydroxyacetone and phenylglyoxal are oxidisable by the very dilute H_2O_2 without peroxidase catalysis; the same can now be said of formaldehyde.

Tables I–V give the experimental details of some of the above results.

The amount of enzyme used in all experiments after Table I is about half of what was used in the previous paper, the smaller amount being used to economise with the enzyme preparations and to diminish the slight destruction of H_2O_2 by catalase. The experiments were all done under roughly the same conditions of H_2O_2 concentration, p_H and temperature as in the ordinary purpurogallin method of estimation. Calculating, therefore, from the rate of H_2O_2 usage in the purpurogallin method, this smaller amount of enzyme would be 2250 times enough to use up all the H_2O_2 in the time of the experiments if the enzyme worked equally rapidly with other reducing substrates. Insufficiency of enzyme, therefore, cannot account for the negative results obtained.

Table I.

Each flask contained 1·15 P.E.* of enzyme (*i.e.* 12 mg. of P.Z. 95) in 20 cc. phosphate buffer p_H 6·4, and the neutralised substrate in 5 cc. H_2O. 10 cc. of 0·02 % H_2O_2 were added over 45 minutes. Total time 1 hour before removing 1 cc. sample and adding acid. Temperature 20°.

	p-Phenylene-diamine 50 mg.	Tyrosine 50 mg.	Trypto-phan 50 mg.	Histidine-HCl 50 mg.	Controls	
					Water	Water
Colour formed	Blue	Yellow	–	–	–	H_2O_2 all added together and acid at once
Sample: Colour with *p*-phenylene-diamine	.	–	+ +	+ +	+ +	.
Sample: Do. *plus* H_2O_2	.	+ + + +	+ + + +	+ + + +	+ + + +	.
H_2O_2 remaining at end. (cc. O_2 evolved by MnO_2)	0·06	0·0	0·67	0·66	0·57	1·61

* P.E. (=Purpurogallin Einheit) is the usual abbrevation for Purpurogallin Unit as defined by Willstätter and Pollinger [1923].

p-Phenylenediamine and tyrosine use up H_2O_2 and are therefore oxidised. Tryptophan and histidine are not oxidised. The enzyme is always still active at the end of the experiment.

Table II.

As for Table I except that each flask contained only 0·64 P.E. of enzyme, *i.e.* 6·7 mg. of P.Z. 95.

		Glycine 25 mg.	Glutamic acid 50 mg.	Acetic acid 25 mg.	Sodium lactate 50 mg.	Alcohol 25 mg.	Controls: Water	Controls: All H_2O_2 added together and acid at once
Sample	Colour with *p*-phenylenediamine	+ +	+ +	+ +	+ +	+ +	+ +	.
	Do. *plus* H_2O_2	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	.
cc. O_2 evolved by MnO_2 at end		0·87	0·82	0·87	0·86	0·84	0·87	1·45

None of the above compounds is oxidised. The enzyme is active at the end of all experiments.

Table III.

0·63 P.E. of enzyme in each flask.

		Formic acid 25 mg.	Glucose 50 mg.	Formaldehyde 30 mg.	Glycerol 40 mg.	Acetaldehyde 40 mg.	Water
Sample	Colour with *p*-phenylenediamine	+ +	+ +	(+)	+ +	+ +	+ +
	Do. *plus* H_2O_2	+ + +	+ + +	+ +	+ + +	+ + +	+ + +
cc. O_2 evolved by MnO_2		0·92	0·80	0·14	0·86	1·16	0·85

Formic acid, glucose, glycerol, and acetaldehyde are not oxidised. The curious effect of rather greater liberation of O_2 in the presence of acetaldehyde than in its absence occurred also with milk-peroxidase. I have shown by experiments that acetaldehyde in acid solution yields no gas with MnO_2 nor is extra gas yielded on decomposing H_2O_2 when acetaldehyde is present. One can only assume that the aldehyde protects the H_2O_2 to some extent from the slight decomposition by catalase during the experiment.

Formaldehyde used up the hydrogen peroxide but separate experiments showed that this occurred to the same extent in the absence of any enzyme.

Table IV.

		Brucine 60 mg.	Fructose 90 mg.	Oleic acid 50 mg. in neutral emulsion	Tannin 100 mg. Dark green colour formed	Water
Sample	Colour with *p*-phenylenediamine	+ +	+ +	+ +	−	+ +
	Do. *plus* H_2O_2	+ + +	+ + + +	+ + + +	+ +	+ + + +
cc. O_2 evolved by MnO_2 at end		0·66	0·63	0·62	0·0 In neutral solution	0·56

Table V.

		Phenyl-alanine 50 mg.	Tyrosine 25 mg. powdered	Catechol 25 mg.	Gallic acid 40 mg.	Water
Colour formed		–	Yellow	Deep yellow	Dark greenish brown	–
Sample	Colour with *p*-phenylenediamine	+ +	–	–	–	+ +
	Do. *plus* H_2O_2	+ + + +	+ + + +	+ + + +	+ + +	+ + + +
cc. O_2 evolved by MnO_2 at end		0·97	0·0	0·0	0·15	1·01

Phenylalanine is not oxidised while tyrosine, catechol, and gallic acid are.

Phenols. Tyrosine is acted upon by H_2O_2 and peroxidase and it has long been known that various other phenols are oxidised, the reactions with them having been used as tests for the enzyme [see Raper, 1928]. Experiments were made with a representative series of phenolic substances with the result that one may say that all substances containing a phenolic group are oxidisable by H_2O_2 under the influence of catalysis by both horseradish- and milk-peroxidase. Only one exception has been found, namely resorcinol, which, though oxidised by the plant-enzyme, completely inhibits the activity of the milk-enzyme; it is not oxidised itself and in small amount prevents the oxidation of iodide, benzidine, *p*-phenylenediamine, *etc.*

Table VI gives a summary of the observations on phenols. In this table under "qualitative" are given the observations in simple test-tube experi-

Table VI.

	Plant-peroxidase		Milk-peroxidase	
	Qualitative	Quantitative	Qualitative	Quantitative
Phenol	Yellow		Yellow	
o-Cresol	Greenish-yellow +ppt.		Greenish-yellow	
p-Cresol	White ppt.		–	+
m-Cresol	White ppt.	+Faint yellow	–	+Faint yellow
Vanillin	White ppt.	+Faint yellow	–	+Yellow
Tyrosine	–	+Yellow	–	+Yellow
Tyramine	–	+	–	+
Catechol	Yellow	+Deep yellow	Yellow	
Resorcinol	Brownish-yellow		Inhibition	
Quinol	Pale yellow		Pale yellow	
Protocatechuic acid	Yellow		Yellow	
3 : 4-Dihydroxy-phenylalanine	Red-brown		Red-brown	
Adrenaline	Red		Red	
Pyrogallol	Yellow		Yellow	
Gallic acid	Green-brown	+Colour	Green-brown	
Tannic acid	Dirty yellow	+Colour	Dirty yellow. Enzyme largely precipitated with protein	+Colour
α-Naphthol	Magenta +ppt.	+Colour	Magenta	+Colour
β-Naphthol	White ppt.		–	+Faint yellow

– signifies "nothing easily observable." + signifies oxidised.

ments. A number of the substances, especially with the milk-enzyme, showed no marked colour or precipitation reaction; these were therefore tested by the quantitative method of estimating H_2O_2 usage, and a number of the others were confirmed by this method. The results are shown under the heading "quantitative."

No attempt has been made to discover the products of reaction.

Tables VII–VIII give the details of some of these "quantitative" experiments.

Table VII.

Horseradish-peroxidase. 0·66 P.E. in each flask.

	Tyramine 40 mg.	Gallic acid 40 mg.	α-Naphthol 40 mg.	Vanillin 40 mg.	*m*-Cresol 40 mg.	Water
Colour formed	?Scarcely detectable	Violet going at once green-brown	Light yellow with white ppt.	Faint yellow white ppt.	Light yellow milky white ppt.	–
Sample: Colour with *p*-phenylenediamine	–	–	–	–	–	++
Sample: Do. *plus* H_2O_2	+++	+++	+++	++++	++++	++++
cc. O evolved by MnO_2 at end	0·0	0·15 In neutral solution	0·0	0·0	0·0	0·81

Table VIII.

Milk-peroxidase. 0·7 P.E. in each flask.

	Tyramine 40 mg.	α-Naphthol 40 mg.	β-Naphthol 40 mg.	Vanillin 50 mg.	*m*-Cresol 50 mg.	*p*-Cresol 40 mg.	Tannic acid 40 mg.	Water
Colour formed	Scarcely detectable	Magenta	Faint yellow	Faint yellow	Faint yellow	White opalescence	Yellow-brown	.
Sample: Colour with *p*-phenylenediamine	–	–	–	–	–	–	–	+++
Sample: Do. *plus* H_2O_2	+++	+++	+++	+++	++	+++	(+)*	+++
cc. O_2 evolved by MnO_2 at end	0·0	0·0	0·0	0·0	0·0	0·0	0·39	0·96

* Considerable precipitation of enzyme had taken place.

Experiment showed that none of these substances took up H_2O_2 in the absence of enzyme; all of them are therefore oxidised under the influence of catalysis by the enzyme.

The determination of unused H_2O_2 in the experiments with tannin and gallic acid by the MnO_2 method had to be made in neutral solution since it was found that acid solutions of these substances, and of pyrogallol, evolve an uncertain amount of gas (oxygen?) when shaken with MnO_2 in the absence of any H_2O_2.

Dilute iron solutions (0·1 mg. of $FeSO_4$, $7H_2O$ or $FeCl_3$) with dilute H_2O_2 oxidised some of the above phenols slowly, but in no case was the reaction anything like as strong as with the enzyme solutions.

Pyrrole was tried (50 mg. and 15 mg. in the usual volume) and found not to be oxidised with milk- or horseradish-peroxidases. Its presence causes probably complete inhibition of the milk-enzyme and very great inhibition of the plant-enzyme. With pyridine also, some degree of inhibition of the activity of both enzymes was observed.

Discussion.

The following is a list of the substances shown, in this and my last paper and by previous workers, to be oxidised by H_2O_2 under the catalysis of the peroxidases of milk and horseradish. The numerous compounds of various types tried and found not to be oxidised by the system are listed elsewhere.

Milk-peroxidase.

Iodide, nitrite, tryptophan.
Diamines such as *o*- and *p*-phenylenediamine, benzidine, *etc.*
Guaiacum and all phenolic substances (except resorcinol which inhibits the enzyme).

Horseradish-peroxidase.

Iodide.
Diamines such as benzidine, phenylenediamines, leuco-malachite green, *etc.*
Guaiacum and all phenolic substances.

Studies of these two very similar enzymes have brought to light a number of interesting differences between them which should have some bearing on theories of the mechanism of their actions. The difference in p_H range of activity mentioned in the last paper is probably a function of the accompanying colloidal material in the preparation; the inhibition by resorcinol of the milk-enzyme, but not of the plant preparation, may possibly also have something to do with the colloidal states of the two preparations. But the oxidations of nitrite and tryptophan by the one and not by the other seem to be real differences between the active systems. Also a glance at Table VI suggests that the products of oxidation are not always the same in the two cases. As to the specificity towards the oxidising substrate, Wieland and Sutter [1930] have studied the activation of various organic peroxides by horseradish-peroxidase and it would be interesting to see their work repeated with the milk-enzyme. Dixon [1929] has stated that milk- but not horseradish-peroxidase will use persulphate as oxidising substrate.

The theory of the mechanism of peroxidase reaction put forward by Mann [1931] could perhaps explain these differences by asssuming differences in the specificity of the linkages combining the two substrates to the enzyme.

The present extension of my last paper has not thrown any further light on the place of peroxidase as a constituent of the respiratory mechanism. If a function is to be found for peroxidase it may be, as suggested before, that in its normal environment the enzyme is provided with substrates "activated" by other catalysts; its ready ability to form coloured oxidation products may be of importance, though the non-oxidation of, and inhibition by, pyrrole does not seem to support this in the case of animal tissues.

In view of the general reaction demonstrated with phenols, some revision of the present ideas concerning the interrelation of oxidases and peroxidases in plant tissues may be necessary.

Summary.

1. The preparation of horseradish-peroxidase in fairly concentrated form is described. The method consists simply in extraction of the minced root with water, precipitation of the enzyme by saturation with ammonium sulphate, purification of the precipitate by dialysis and filtration, and finally fractional precipitation by alcohol.

2. Using the method described by Elliott [1932] it was shown that nitrite and tryptophan are not oxidisable by H_2O_2 with the plant-enzyme, which is therefore different from the milk-peroxidase.

3. The following substances have also been tried with the horseradish-peroxidase and found not to be oxidised: formate, acetate, lactate, oleate, glucose, fructose, glycerol, ethyl alcohol, acetaldehyde, glycine, glutamic acid, phenylalanine, histidine, brucine.

4. Formaldehyde, like dihydroxyacetone and phenylglyoxal as shown previously, is oxidised by very dilute H_2O_2 without enzyme.

5. Positive results with a representative series of phenols allow the conclusion to be drawn that all phenolic substances are oxidised by H_2O_2 with both horseradish- and milk-peroxidases.

6. To the above generalisation one exception was found. Resorcinol, though oxidised with the plant-enzyme, is not oxidised by milk-peroxidase the activity of which it inhibits.

7. Pyrrole is not oxidised with either enzyme and also has an inhibitory action which appears more complete with the milk-enzyme. Pyridine also has some inhibitory effect on the action of both enzymes.

It is a pleasure for me here to record my gratitude to Geheimrat H. Wieland for allowing me to work in his department, and for his kind interest in this work. I am also grateful to Sir F. G. Hopkins for his encouragement.

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CLIII. A POLYSACCHARIDE FROM THE POLLEN OF TIMOTHY GRASS (*PHLEUM PRATENSE*).

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Recent investigations of the relationship of various constituents of pollens to the allergic diseases for which they are responsible have indicated that, in most cases, the active principles are associated with the protein fractions. In an examination of the fractions of Timothy grass pollen an attempt was made to isolate a polysaccharide on account of the possibility that such a compound might play a part similar to those of the specific soluble carbohydrates isolated from various pathogenic bacteria.

By use of the methods employed in the isolation of the specific carbohydrate from the tubercle bacillus it has been possible to isolate from the pollen a laevorotatory complex carbohydrate which appears to be of the same chemical type as the corresponding bacterial substances. A preliminary chemical examination has shown that it yields *l*-arabinose, galactose, and a non-reducing acid on hydrolysis with acids, thus displaying a certain similarity to the specific carbohydrate of the tubercle bacillus. The relationship of the polysaccharide to the allergic reactions of the protein fractions of the pollen is being examined.

It is improbable that the polysaccharide is in any way connected with the glucoside dactylin, isolated by Moore and Moore [1931] by direct aqueous extraction of Timothy and Orchard grass pollens, since dactylin gives no pentose reactions. There is very little recorded concerning the occurrence of gum-like carbohydrates in other pollens; Johnson and Rappaport [1932] state that no such substance could be isolated from ragweed pollen, whilst Miyake [1922] describes the isolation of 5·7 % of a pentosan from maize pollen. Black [1931] isolated from the pollen of the giant ragweed a carbohydrate containing 5·2 % N which reacted specifically in the skin and nasal mucosa of ragweed-sensitive persons.

Experimental.

Preparation of the carbohydrate.—10 g. of pollen (*Phleum pratense* collected in 1930) were digested with 4 % sodium hydroxide (200 cc.) on the water-bath for 6 hours. At the end of this period some of the cells were broken, but most were only swollen and slightly ruptured. The liquid was diluted with an equal

volume of water, acidified with acetic acid and filtered. The filtrate was treated with an excess of lead acetate and the precipitate, which contained most of the yellow colouring matter but gave no Molisch reaction, was removed and discarded. Basic lead acetate and sufficient ammonia to basify the liquid strongly were then added and the heavy precipitate, which contained all the substance responsible for the Molisch reaction, was removed, washed with water and suspended in dilute acetic acid. After stirring mechanically for several hours the insoluble material was removed and discarded and the filtrate treated with hydrogen sulphide. The lead-free liquid was then evaporated at 40° to a small volume and poured into ten volumes of ethyl alcohol. The precipitated solid was dissolved in water (8 cc.), treated with an equal volume of methyl alcohol and the precipitated material filtered off. When thus isolated, the material insoluble in 50 % methyl alcohol was only slightly soluble in cold and somewhat more so in hot water. It gave no coloration with iodine and did not reduce Fehling's solution. Upon hydrolysis with acids reducing sugars were formed.

The water-soluble carbohydrate was obtained by pouring the filtrate from the above material into an excess of methyl alcohol (80 cc.) and was further purified by another precipitation from 50 % methyl alcohol.

Two other preparations were made, one from pollen (30 g.) which had been ground in a ball-mill and extracted with dilute saline and the other from pollen (55 g.) which had previously been extracted with acetone. The yields were 1·1 and 1·9 g., respectively. In the last preparation the treatment with sodium hydroxide was prolonged until most of the pollen grains had burst (32 hours).

The preparations formed white powders which were very soluble in water and which changed to gummy masses on addition of a few drops of water. The last two preparations had respectively $[\alpha]_{5461}$ − 42·0° and − 39·1° in 1 % aqueous solution. A fractional precipitation experiment was made to test the homogeneity of the material; a solution of the third preparation (0·2 g.) in water (1·5 cc.) was treated with alcohol (4·5 cc.), the precipitated material (0·06 g.) removed, the filtrate poured into an excess of alcohol (60 cc.), and the second fraction collected (0·13 g.). The first fraction had $[\alpha]_{5461}$ − 34·2° and the second − 39·1°. The fractions contained 0·9 and 0·5 % N. The unfractionated preparation contained 0·2 % P.

The carbohydrate gave strong characteristic reactions for pentoses with hydrochloric acid and phloroglucinol and with hydrochloric acid and orcinol. No reaction for methylpentoses could be obtained by the reaction of Rosenthaler [1909]. No reaction for glycuronic acid was obtained using the modified method of Neuberg and Kobel [1931]. The carbohydrate possessed only a very faint reducing power towards Fehling's solution, but after warming for a few minutes with dilute hydrochloric acid much reducing sugar was formed.

An experiment was made on the rate of hydrolysis of the carbohydrate in sulphuric acid in order to determine whether the sugars were liberated from the complex in stages or not. 7·908 mg. of the carbohydrate were dissolved in 1 % sulphuric acid (200 cc.) and 10 cc. portions of this solution were heated in

a boiling water-bath for periods up to 4½ hours. At the end of the chosen period each tube was cooled quickly and immediately neutralised with the appropriate amount of *N* sodium hydroxide. The amounts of reducing sugars formed were determined as glucose by the Hagedorn-Jensen method.

Tube	Time mins.	% Reducing sugar formed
1	0	4·5
2	5	12·4
3	10	32·6
4	12	34·9
5	20	36·2
6	40	42·8
7	60	45·3
8	80	45·8
9	120	46·5
10	160	48·3
11	220	52·4
12	260	50·1

The values obtained show that reducing sugars are rapidly formed during the first hour of hydrolysis and that after that time a further slow formation of reducing substances occurs which ceases after about four hours.

For the purpose of examination of the products of hydrolysis, 1·1779 g. of the carbohydrate were heated on the water-bath for 2 hours in 100 cc. of 1 % sulphuric acid. The observed rotation ($\lambda = 5461$, $l = 1$ dm.) changed during this time from − 0·46° to + 0·64°. The liquid was then digested with an excess of barium carbonate until neutral to litmus paper. After filtration the liquid, which contained barium ions, was evaporated to a small volume and poured into ten volumes of methyl alcohol. The precipitated material (0·18 g.; Ba, 27·0 % on air-dried material) did not reduce Fehling's solution and gave no naphthoresorcinol reaction. The methyl alcohol-soluble portion was evaporated to a small volume and again treated with methyl alcohol; a trace of insoluble material was removed. The methyl alcohol was finally removed and the residue evaporated several times with water. The residual syrup gave the deep red colour characteristic of pentoses upon distillation with 12 % hydrochloric acid and pouring the distillate into a solution of aniline acetate. Attempts were made to form hydrazones with the following hydrazines: phenylhydrazine, *as*-phenylmethylhydrazine, *as*-benzylphenylhydrazine, *p*-bromophenylhydrazine, and *as*-diphenylhydrazine. Phenylhydrazine gave a small amount of a substance freely soluble in water and crystallising in large needles; it appeared to be quite distinct from mannose phenylhydrazone; *p*-bromophenylhydrazine and phenylmethylhydrazine gave clusters of fine needles similar in appearance to the galactose compounds. Diphenylhydrazine and benzylphenylhydrazine gave comparatively large amounts of crystalline products. More of the diphenylhydrazone was prepared and washed with alcohol and water. It had M.P. 197–199° (decomp.) whilst a sample of authentic *l*-arabinose diphenylhydrazone melted at 199–202° (decomp.); Müther and Tollens [1904] give M.P. 204–205° (decomp.). A solution of this diphenylhydrazone (0·0216 g.) in

pyridine (2 cc.) and alcohol (3 cc.) had $\alpha_{5461} + 0{\cdot}36°$ ($l = 2$ dm.), whilst the authentic specimen (0·0212 g.) had + 0·34° under the same conditions.

Since the appearance of the phenylmethylhydrazone suggested the presence of galactose a portion of the sugar was oxidised with 25 % nitric acid and a small amount of mucic acid was obtained (M.P. 210–212° decomp. when the tube was inserted at 200° and the bath heated fairly rapidly). Oxidation of the original carbohydrate (0·1 g.) also yielded mucic acid (0·0104 g.)

The barium salt (0·1 g.) was subjected to further treatment with 2*N* sulphuric acid (10 cc.) for 16 hours. A small amount of reducing sugar (0·05 g.) was formed and some barium salt recovered (0·03 g.) which gave no naphthoresorcinol reaction. The sugar fraction was divided into two portions one being treated with diphenylhydrazine and the other oxidised with nitric acid. A small amount of mucic acid was obtained, but no arabinose diphenylhydrazone could be isolated.

The recovered barium salt lost 8·17 % of its weight on drying at 110° and contained 30·2 % of barium (calculated on the dried material).

Upon concentration of the alcoholic filtrate from the first precipitation of the carbohydrate a small amount of crystalline material separated in a form which suggested that it might be inositol. After recrystallisation of this substance (0·02 g.) from a small amount of aqueous alcohol it had M.P. 223° alone or mixed with an authentic specimen of inositol.

SUMMARY.

The preparation of a gum-like polysaccharide from the pollen of Timothy grass is described. Upon hydrolysis with acids *l*-arabinose, galactose and a non-reducing acid are produced.

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CLIV. THE PRODUCT OF THE OXIDATION OF GLUCOSE BY GLUCOSE DEHYDROGENASE.

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(*Received July 1st, 1932.*)

Recently, a description was given of the preparation from the livers of various animals of a new enzyme, glucose dehydrogenase, which brought about the oxidation of glucose, apparently with the formation of an acid [Harrison, 1931]. The present paper deals with the isolation and identification of this oxidation product.

Glucose in presence of the dehydrogenase is not oxidised by molecular oxygen, but is oxidised in presence of methylene blue. It was shown that the aerobic oxidation of glucose could be brought about by the dehydrogenase if the oxygen-activating system cytochrome and indophenol oxidase were present. Since it was difficult to prepare sufficient cytochrome for a large scale oxidation of glucose, the oxidation was carried out in presence of air, using methylene blue as an intermediary carrier. The methylene blue is reduced to leuco-methylene blue which is then rapidly oxidised back to methylene blue by the oxygen of the air. The methylene blue thus acts as a carrier between the activated hydrogen of the glucose and the oxygen of the air.

In order to obtain a clue as to the nature of the oxidation product, a number of preliminary experiments were carried out in the Barcroft differential manometer to determine the relationship between the amount of acid formed and the amount of oxygen taken up. The dehydrogenase preparation and methylene blue were put into both vessels of the apparatus, and glucose solution was added to one vessel and an equal volume of water to the other vessel. The enzyme was the dialysed preparation from acetone-liver [Harrison, 1931], equivalent to about 5 g. of acetone-liver in each vessel. The final concentration of methylene blue was 1 in 2500 and of glucose 0·25 *M*. After shaking for about 3 hours at 37°, the oxygen uptake was measured, and the solution in the vessel containing glucose was titrated with $N/50$ NaOH to the same p_H as that of the solution containing no glucose. In spite of the presence of methylene blue, this could be carried out sufficiently accurately by withdrawing a very small amount of solution on the end of a glass rod and using an external indicator such as phenol red. The titration was checked electrometrically using hydrogen electrodes.

As the result of a number of determinations, an average ratio of slightly less than one atom of oxygen to one equivalent of acid formed was obtained. This rendered improbable the formation of a number of acids such as glycuronic,

keto-gluconic or saccharic acid, all of which require more than one atom of oxygen per equivalent of acid. Determination of lactic acid in the solutions with and without glucose had already shown that no lactic acid is formed from glucose during the oxidation [Harrison, 1931]. Experiments on a rather larger scale showed that after removal of protein from the solution, negative nitroprusside and guaiacol tests were obtained for pyruvic acid, and a negative naphthoresorcinol test was obtained for glycuronic acid, though strongly positive tests were given on adding traces of these acids to the solution.

By comparing the amount of precipitate in the solutions with and without added glucose, it was found that the oxidation product of glucose was precipitated by basic lead acetate but not by normal lead acetate. This again excluded as main products of the reaction a number of acids such as malic, tartaric, citric, succinic, malonic and glyceric acids.

On decomposing the basic lead acid precipitate and treating the liberated acid with $CaCO_3$, a calcium salt was obtained which was soluble in water but which was precipitated by alcohol. The acid did not reduce Fehling's solution but reduced hot ammoniacal silver nitrate. In view of the elimination of the acids mentioned above, these properties, coupled with the observation that one equivalent of acid is formed for each atom of oxygen taken up, suggested that gluconic acid might very possibly be the oxidation product. By carrying out the oxidation on a large scale, it was found possible to prepare and isolate derivatives of the acid which were found to be identical with the corresponding derivatives of *d*-gluconic acid.

The methods used for isolation differed somewhat in different experiments, but the technique used in the experiment to be described proved to be the most satisfactory, and details of the other experiments will therefore be omitted.

The glucose dehydrogenase was prepared by water extraction of 450 g. of acetone-liver, followed by dialysis and precipitation by saturation with ammonium sulphate exactly as previously described [Harrison, 1931]. The dry preparation was ground up, put into two large wet collodion sacs and dialysed into solution against distilled water in the ice-chest. After adjusting to p_H 7·4 and centrifuging, the volume of the enzyme solution was 130 cc.[1] 125 cc. of this solution were mixed with 25 cc. of 2*M* glucose (B.D.H. dextrose) and 37·5 cc. of methylene blue (1 in 500), the mixture being put into corked 250 cc. conical flasks and shaken fairly rapidly in a water-bath at 37°. The flasks were opened at frequent intervals and filled with fresh air.

At the same time, two Barcroft differential manometers were used to measure the oxygen uptake. The right-hand vessels each contained 2 cc. enzyme and 0·6 cc. methylene blue. The experimental apparatus contained in addition 0·4 cc. 2*M* glucose, while the control apparatus which measured the residual oxygen uptake of the enzyme preparation received 0·4 cc. water. The side tubes

[1] Recent experiments by Mann [1932] have shown that an activator is concerned in the oxidation of glucose by glucose dehydrogenase. The enzyme preparation described above contains both the dehydrogenase and the activator.

of the vessels contained NaOH to absorb CO_2, and the vessels were shaken in the same water-bath and for the same length of time as the conical flasks.

After four hours, the oxygen uptake in the Barcroft apparatus was measured, and the flasks were opened. Incubation of samples on agar plates showed that the solutions were almost sterile and that the oxidation of glucose was therefore not due to bacteria.

The solutions in the flasks, together with the contents of the Barcroft vessel containing glucose, were brought to approximately p_H 7·6 and the protein was precipitated by the addition of absolute alcohol to a concentration of 80 %. After filtering under suction, the residue was ground up with 100 cc. of water, filtered and washed. The latter filtrate was precipitated with 80 % alcohol and filtered, using a trace of kieselguhr on the filter-paper. The precipitate was ground with a little water, acidified to p_H 4 with acetic acid, filtered, precipitated with 80 % alcohol and again filtered. All the alcoholic filtrates were combined and evaporated down under reduced pressure to about 150 cc.

The solution was brought to p_H 7·6, allowed to stand overnight to permit of the hydrolysis of any lactone, and precipitated by adding a saturated solution of basic lead acetate together with a little NaOH until a drop of either produced no further precipitate (p_H approximately 7·8). The precipitate was suspended in water, stirred well with acetic acid and then brought almost to neutrality by adding NaOH. The precipitate, consisting largely of $PbSO_4$ (from $(NH_4)_2SO_4$ contained in the enzyme preparation) was centrifuged off, washed, and the clear solution and washings were made alkaline and reprecipitated with basic lead acetate. The precipitate was washed twice with water containing a little basic lead acetate. All the washings and the solution left from the first precipitate were combined, freed from lead with H_2SO_4 and $Ba(OH)_2$, concentrated under reduced pressure and reprecipitated with basic lead acetate. (The solutions were never allowed to stand in presence of basic lead acetate in order to avoid the risk of decomposition of any glucose in the solution.) The combined basic lead acetate precipitates were suspended in water and treated with slight excess of H_2SO_4 to remove lead, the excess of H_2SO_4 being exactly removed with baryta. The clear filtrate was evaporated to dryness over solid NaOH in a vacuum desiccator, and most of the acetic acid was removed by reducing the pressure to about 0·5 mm. Hg. (The methylene blue had been almost completely removed at this stage since it was not adsorbed on the lead precipitate.) The residue was dissolved in a little water, stirred with 0·2 g. $CaCO_3$ and left overnight over NaOH. The mixture was then heated on the water-bath for several hours, stirring at intervals, and when nearly neutral a few drops of lime water were added until the solution was permanently just neutral to phenol red. The solution was filtered and the calcium salt was precipitated with 80 % alcohol in a total volume of about 50 cc. After standing overnight in the ice-chest, the precipitate was centrifuged off and dissolved in about 2 cc. of warm water, and warm alcohol was added to a concentration of 30 %. On standing overnight the calcium salt crystallised out and was filtered and washed with a little

cold 30 % alcohol and dried over H_2SO_4. The mother-liquor and washings were evaporated down and again allowed to crystallise. By repeating this process four times, assisting crystallisation each time by seeding the solution with a crystal from the previous crop, a total of 255 mg. of crystalline salt was obtained, the crystals consisting of clusters of microscopic needles resembling the highly characteristic crystals of the calcium salt of gluconic acid.

By heating a portion of the free acid on the water-bath with phenylhydrazine and acetic acid [Fischer and Passmore, 1889], crystals were obtained on cooling which, after dissolving in hot water, decolorising with a little charcoal and recrystallising once from very dilute acetic acid and three times from hot water, commenced to decompose at 196° on rapidly heating and melted completely at 200°. A sample of the phenylhydrazide of gluconic acid prepared from an authentic specimen of the acid melted at the same temperature, and the melting-point was not changed by mixing the two specimens together. The two samples of crystals were apparently identical under the microscope, being microscopic, square-ended prisms with straight extinction. Further confirmation that the acid is gluconic acid was obtained by preparing the cinchonine salt which after recrystallising from 95 % alcohol had M.P. 185–186°. Micro-analysis (Schoeller) of the phenylhydrazide: C, 50·38, 50·50; H, 6·22, 6·28 %. (Calculated for $C_{12}H_{18}O_6N_2$: C, 50·35; H, 6·29 %.)

There appears to be no doubt, therefore, that gluconic acid is formed by the oxidation of glucose in presence of glucose dehydrogenase.

In order to determine whether the acid were *laevo-* or *dextro*-gluconic acid, 150 mg. of the dry, recrystallised Ca salt were dissolved in water, made up to 5·0 cc., and the rotation measured in a 1 dm. tube at 20°. Taking the average of a number of closely agreeing readings, the specific rotation $[\alpha]_D^{20^\circ}$ was calculated to be + 8°. The values given in the literature for Ca *d*-gluconate range from + 6° to + 10°. As would be expected, therefore, the *dextro*-form of gluconic acid is formed by the oxidation of *d*-glucose by the enzyme.

The oxygen uptake during the oxidation was equivalent to the formation of 424 mg. of calcium gluconate (after subtracting the residual oxygen uptake of the enzyme alone). The amount actually isolated in the crystalline state was 255 mg. which is equivalent to just over 60 % of the oxygen uptake observed. In dealing with such small amounts, the isolation is by no means quantitative, and it seems quite likely, therefore, that *d*-gluconic acid is the sole product of this oxidation of glucose.

Discussion.

The oxidation of glucose at the potential aldehyde group with the formation of gluconic acid is readily brought about by a number of oxidising agents and would seem at first sight a probable method of breakdown of glucose in the body. The results reported in the literature of feeding and perfusion experiments with gluconic acid are rather contradictory, but there appears to be a certain amount of evidence that some utilisation of gluconic acid takes place. Experi-

ments in which unchanged gluconic acid was recovered in the urine after feeding the acid may possibly be explained by the rapid elimination of the acid by the kidneys after feeding in relatively large doses, this preventing a quantitative utilisation of the acid.

While, therefore, gluconic acid may be a normal intermediary in the oxidation of glucose, an alternative possibility might be that the gluconic acid which has been isolated in these experiments may arise from some labile oxidation product of glucose which normally, in the intact cell, becomes further oxidised instead of appearing as gluconic acid. A similar hypothesis has been brought forward by Meyerhof [1930] to explain the conversion by muscle enzyme of glycogen to lactic acid *via* hexosemonophosphate. In this case, although hexosemonophosphate can be isolated during the reaction, yet glycogen is broken down by the enzyme more rapidly than the supposed intermediary, hexosemonophosphate. Meyerhof explains this by assuming that the true breakdown path is *via* an unknown labile form of hexosemonophosphate which, if not immediately converted into lactic acid, may appear as stable hexosemonophosphate.

I put these possibilities forward only as mere suggestions, but in view of the experiments I have described and of the high concentration of glucose dehydrogenase in the liver, it seems difficult to escape from the conclusion that in the body, part, at least, of the glucose metabolised must go through gluconic acid or some precursor of this acid during its oxidation.

It is interesting that glucose dehydrogenase and glucose oxidase, the enzyme prepared from *Aspergillus niger* by Müller [1928], both convert glucose into gluconic acid; for, as previously pointed out [Harrison, 1931], the two enzymes differ fundamentally in properties, the oxidase being able to utilise oxygen but not methylene blue as the hydrogen acceptor, while the dehydrogenase can use methylene blue but not oxygen directly.

SUMMARY.

The product of the oxidation of glucose by glucose dehydrogenase has been isolated and shown to be *d*-gluconic acid.

The amount of acid isolated as the crystalline calcium salt was equivalent to over 60 % of the amount formed as calculated from the oxygen uptake, and *d*-gluconic acid is probably the sole product of the oxidation.

The author wishes to express his thanks to Prof. E. Mellanby for his interest in this work.

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CLV. CASTRATED RATS FOR THE ASSAY OF TESTICULAR HORMONE.

By VLADIMIR KORENCHEVSKY.

From the Lister Institute, London.

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In a previous paper[1] [Korenchevsky, 1932] the increase in weight of the prostate with seminal vesicles of injected castrated rats by at least 40 % as compared with those of control uninjected rats was proposed as a quick and sensitive method of assay of testicular hormone.

Since, after castration, the weights of the penis and thyroid also decrease, whilst the hypophysis, adrenals and body fat increase in weight, the suggestion was made that, for a test of the complete activity of the testicular hormone, it was necessary to obtain a return towards normal of these organs.

The importance for accurate assay of using litter-mate castrated rats was stressed. In this case the litters were to be divided into three groups, (1) controls uninjected, (2) rats injected with a standard preparation, or a previously prepared preparation, (3) rats injected with the new preparation of hormone. The chief difficulty in the assay is that, up to the present time, such a standard preparation is not available. Even if it were available it would with difficulty be kept at a constant strength owing to the instability of the testicular hormone, due chiefly to oxidation. In any case it would be necessary to test the standard preparation from time to time.

The question, therefore, arises under what conditions the castrated rats of different litters will give the nearest comparable results in assay.

In the previous paper was noted the importance of using rats in litters from the same stock, reared on the same diet, of the same age, at the same date after castration, and kept during the experiment on the same complete synthetic diet.

The present communication contains a study of the post-castration changes of the organs mentioned above in different litters of male rats, in relation to sexual maturity at the time of castration of the rats and to the number of days elapsing between castration and the examination of the rats.

[1] On p. 420 of this paper, the weights of adrenals, thyroids and hypophysis must be divided by 10, the decimal points having been inadvertently omitted.

PRESENT INVESTIGATION.

Eighty-eight castrated rats belonging to 48 litters (40 litters in pairs and 8 litters singly) have been arranged in Tables I and II according to sexual maturity at the time of castration and the interval in days between castration and the time at which the rats were killed. These rats were used as the controls of several other experiments. All the rats belonged to the Lister Institute stock and were reared on the same diet.

The 50th day after birth was chosen as the age of sexual maturity. In the Lister Institute, sexual maturity, as judged by fertility, has been exceptionally noted as early as the 38th day after birth, and frequently, but not generally, at the 50th to 56th day (unpublished results of Miss H. H. Smith). The 50th day was, therefore, taken as the earliest frequent date of sexual maturity. This is in agreement with most observations on different stocks. Allen [1918], using rats from the Wistar Institute, found the first ripe spermatozoa in testes between the 36th and 40th day; Andersen [1932] gives the age of sexual maturity as about 60 days as judged by the finding of motile spermatozoa in the epididymis. The rats, however, about 50 days old sometimes had large numbers of non-motile spermatozoa. Hewer [1916] alone gives a later date for sexual maturity in his stock, namely, at about 70 days old.

It is clear from Table I that each of the four groups of rats castrated in an immature state, arranged in relation to the number of days after castration, represents a particular age with comparatively small deviations from the

Table I. *The number and age of the castrated male rats in the different groups and their average ages (with the deviations from the average in brackets).*

	No. of days after castration when killed							
	20–30		31–70		71–90		136–182	
	No. of Rats	Age in Days	No. of Rats	Age in Days	No. of Rats	Age in Days	No. of Rats	Age in Days
Castrated when sexually immature	8	58 (+11) (−4)	13	95 (+18) (−15)	6	111 (+12) (−10)	11	203 (+13) (−28)
Castrated when mature	22	88 (+12) (−11)	19	137 (+64) (−36)	8	184 (+12) (−16)	1	286

average age for each group. In the rats castrated after maturity these deviations are greater in the second group. As would be expected, the longer after castration the rats were killed, the older they were.

The weights of the organs were calculated per 200 g. of body weight for each rat. When the litter was represented by a pair of rats the average weight of each organ was found for this litter. From these weights a general average weight of the organs for each group of litters was made and tabulated, together with the maximum deviations from the general average.

Thus the greatest fluctuations in each group between the different litters are shown for each organ. In addition the variations between the different

groups in relation to sexual maturity at castration and the number of days elapsing between castration and the killing of the rats are shown.

In relation to the thymus and penis the following facts must be taken into consideration. The thymus was not dissected in the earlier experiments and, therefore, the averages are obtained for only 28 rats belonging to 14 litters, that is from a small number of observations.

With regard to the penis the technique of dissection of this organ was changed, and in some of the first litters, which unfortunately were not noted, the penis was dissected together with the enlargement at the root by which it is attached to the pelvic bones. Later it was found that the weighing of the penis is much more accurate if the section was made just above these enlargements[1]. The weights by the second method are, therefore, smaller than by the former method. Hence the most reliable data with regard to the sexual organs are those for prostate with seminal vesicles. It is also necessary to note that the fourth group of "mature" litters was represented by one litter only and, therefore, no conclusions can be drawn for this group.

Sexual organs.

From Table II it is clear that the period at which castration is performed, *i.e.* before or after sexual maturity, is of great importance with regard to the

Table II. *The average weights of the organs and retroperitoneal fat per 200 g. of body weight of castrated male rats belonging to different litters in relation to the sexual maturity at the time of castration and the number of days after castration at death (with the maximum percentage deviation from the average in brackets).*

Organs	Condition of rats at castration	Days elapsed between castration and killing: 20–29	30–67	70–89	136–182
Prostate and seminal vesicles (mg.)	Immature	103 (10·7)	60 (26·7)	55 (18·2)	60 (23·3)
	Mature	154 (24·0)	152 (38·1)	180 (16·1)	199 —
Penis (mg.)	Immature	85 (20·0)	90 (52·2)	59 (27·1)	65 (83·1)
	Mature	110 (36·3)	183 (59·5)	221 (10·0)	224 —
Thymus (mg.)	Immature	635 (12·8)	495 (6·3)	390 —	— —
	Mature	447 (29·3)	256 —	— —	— —
Adrenals (mg.)	Immature	66·6 (10·7)	47·4 (27·2)	41·1 (12·9)	42·4 (20·7)
	Mature	46·1 (21·0)	40·0 (41·2)	38·3 (6·5)	43·9 —
Thyroids (mg.)	Immature	22·0 (20·4)	19·8 (42·9)	19·2 (18·2)	17·9 (38·5)
	Mature	20·5 (20·0)	17·8 (47·4)	19·6 (6·6)	15·4 —
Hypophysis (mg.)	Immature	11·8 (11·9)	9·0 (25·5)	8·9 (5·6)	8·6 (11·6)
	Mature	8·7 (25·3)	8·6 (66·3)	8·2 (26·8)	7·1 —
Retroperitoneal fat (g.)	Immature	2·79 (19·3)	6·65 (47·3)	8·00 (19·6)	10·88 (25·9)
	Mature	6·87 (47·0)	7·22 (58·3)	7·34 (29·2)	4·89 —

degree of atrophic changes in the sexual organs. If the rats are spayed before maturity the atrophic changes are greater than if the operation is performed after maturity. At the same time the weight of the prostate with seminal

[1] It has now been found that weighing the dissected penis is more accurate after previous fixation in Bouin's fixative than without fixation (*i.e.* the penis should be treated in the same way as the other organs).

vesicles per 200 g. of body weight remains constant from the end of the first month after castration, being practically the same at six months after castration. During the first month after castration the weight is a little greater.

The fluctuations between the different litters of the same group are not very large, the maximum deviation not exceeding 27 %, and the average deviations being 8·5, 15·0, 10·9 and 15·0 % for the four groups respectively.

If the rats are castrated after sexual maturity, the fluctuations are similar, being again greatest in the second group (31–70 days after castration); the largest deviation was about 38 %, the average deviations being 9·4, 19·3 and 9·4 % for the three first groups respectively.

The somewhat greater fluctuations in the weight of the penis may be due to the use of two methods of dissecting the penis, as mentioned above.

Forty-eight litters yield too small a number of observations from which to select adequate material for the study of the influence of age at castration within the two large divisions grouped as before and after sexual maturity. However, eight litters can be chosen to indicate this latter influence since all were killed at about the same time after castration (from 64 to 69 days after the operation).

1. *Before sexual maturity.* The average weights of the prostates with seminal vesicles of three litters castrated 22–25 days after birth were 46, 50 and 60 mg. per 200 g. of body weight respectively, whilst the average weights of these organs were 76 mg. in each of two litters castrated at 49 days after birth, *i.e.* at a date close to maturity.

2. *After sexual maturity.* These organs in two litters castrated at 64 and 74 days after birth weighed 112 and 126 mg. per 200 g. of body weight, whilst in the litter castrated at 134 days old the weight was 210 mg.

Taking into consideration the fact that maturity may vary from the 36th to the 70th day, as mentioned above, and that the 50th day was taken only as a frequent early date of sexual maturity, some of the rats within this period might actually be operated on before, others during and others after, maturity. Therefore, on the average within this period of age the changes observed may be nearly similar.

The thymus in rats, as in other mammals, reaches its greatest weight at sexual maturity and from then the normal process of involution begins. Castration prolongs the period of growth of the thymus and inhibits involution. In our rats the weight of the thymus per 200 g. of body weight was greater in those castrated before, than in those castrated after, sexual maturity (Table II), which is in agreement with the fact mentioned above.

Retroperitoneal fat increased in amount in the "immature" group with increase in the number of days elapsing between castration and the date of killing of the rats. This difference was not so distinctly marked in the "mature" group.

In the rats killed 20–30 days after castration the weight of fat was greater in the "mature" group (6·87 g.) than in the "immature" group (2·79 g.), which may be due to the greater average age of the rats in the former group.

Adrenals, thyroids and hypophysis. The largest weights per 200 g. of body weight of the adrenals, and to a less degree of the thyroids and hypophysis, of castrated rats were observed in the "immature" group about 20–30 days after castration, gradually decreasing with the increase in number of days after castration. This difference was less marked in "mature" litters. The last group of the latter contained one litter only and the figures must, therefore, be taken with reservation.

The average figures obtained are in good agreement with those previously published [Korenchevsky, 1930, Tables 1–8].

Discussion.

Taking into consideration all the data relating to the changes in weights of organs calculated for the same unit of body weight the following facts are noticeable.

The greatest differences were observed (*a*) between the litters castrated before sexual maturity and those castrated after, and (*b*) between the litters killed during the first month after castration and those killed $4\frac{1}{2}$ months and later after castration.

About $2\frac{1}{2}$ months after operation the changes obtained became more or less stationary (prostate, penis, adrenals) or continued to develop more slowly (fat, thyroids, hypophysis) so that the similarity was found to be greatest in the litters killed $2\frac{1}{2}$–6 months after castration.

The variations in nearly all the organs were greatest in the litters killed 31–70 days after castration, probably owing to uneven rates of development of the changes towards the final state during this period.

Thus the changes produced by castration may be expected to be most nearly similar in different litters if the rats are operated on at the same number of days after birth.

Since the time of operation, before or after sexual maturity, influences profoundly the changes obtained, and since the exact date of maturity in different litters and stocks varies from the 36th to the 70th day, it is advisable to castrate the rats before the 30th day if immature age is chosen for operation, or after the 70th day if sexually mature rats are wanted for operation.

It is also necessary to choose a definite period after castration for beginning the experiment.

Taking into consideration all the limitations mentioned above it must be admitted that in rats belonging to different litters the variations in the weights of the organs investigated were not great provided the rats belonged to the same group.

The inter-relationship between sexual maturity and castration is probably due to the fact that castration before maturity (i) hinders the development and growth of sexual and some other organs, and (ii) produces changes in the already developed organs (*e.g.* atrophy of the sexual organs). After maturity only this latter effect of castration operates.

SUMMARY.

1. Calculated per the same unit of body weight the weights of retroperitoneal fat, sexual and endocrine organs of 88 castrated male rats belonging to 48 different litters were compared.

2. On the average the variations observed between different litters were not great provided the litters belonged to the same group in relation to sexual maturity at the date of castration (before or after sexual maturity) and in relation to the time elapsing after castration.

3. Because of the uncertainty of the exact age of sexual maturity in rats belonging to different litters and stocks, it is advisable to operate on rats before the 30th day after birth if the operations are to be made on immature rats, and after the 70th day if sexually mature animals are to be castrated.

4. In order to obtain accurate and comparable results all the other precautions described in the previous paper must be taken.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled me to carry out this work and to them my thanks are due.

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CLVI. THE RESPONSE OF CASTRATED MALE RATS TO THE INJECTION OF TESTICULAR HORMONE.

By VLADIMIR KORENCHEVSKY, MARJORIE DENNISON and ROSA SCHALIT.

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A method of testing the activity and strength of testicular hormone on castrated rats of the same litter was previously described and conditions were suggested for applying the same method of assay of testicular hormone to rats belonging to different litters [Korenchevsky, 1932, 1]. For this latter purpose it is most important to ascertain (1) how castrated rats belonging to different litters will respond to the same dose of testicular hormone, (2) what conditions must be observed to obtain comparable and accurate results.

In a previous paper [Korenchevsky, 1932, 2] it was shown that the weights of the sexual and endocrine organs and of retroperitoneal fat, when calculated per 200 g. of body weight, did not vary greatly in different castrated litters, bred and kept in the same conditions provided they were operated on at the same period in relation to sexual maturity, *i.e.* before or after puberty, and also that the litters were killed at the same period after castration.

In this paper a study is made of the influence of these two factors on the changes in rats injected with testicular preparations, in order to ascertain under what conditions comparable results for the assay of the hormone when using different litters may be obtained.

The response of the rats of the same litter to different doses was also compared in a few experiments so as to obtain an indication of the sensitivity of the test to differences in the dose.

Technique.

Experiments were performed on 67 rats belonging to 17 litters, all of which came from the stock of the Lister Institute and were reared and kept on the same diet. During the experiments a special paste was given, and all the other details of technique for the assay of testicular hormone were the same as those described elsewhere [Korenchevsky, 1932, 1]. The amount of paste consumed daily was recorded. Castration was performed at different ages in different litters. All the data mentioned above, together with the changes in body weight of the rats, are summarised in Table I.

Table I. *Age and average changes in appetite and body weight of the rats in each group of each litter.*

No. of litter	No. of rats in litter	Age in days: At castration	Age in days: At beginning of injections	No. of days after castration injections started	Change in wt. per 200 g. of initial wt. of rat: Control rats g.	Change in wt. per 200 g. of initial wt. of rat: Injected rats g.	Paste intake per day per 200 g. of body wt.: Control rats g.	Paste intake per day per 200 g. of body wt.: Injected rats g.
1	4	72	82	10	+50	+75	27	28
2	3	70	80	10	+35	+38	20	17
3	4	57	67	10	+92	+84	29	26
4	4	57	67	10	+74	+61	27	25
Average	4	64	74	10	+63	+64	26	24
5	3	25	81	56	+ 7	+14	20	22
6	4	23	79	56	+20	+27	22	20
Average	4	24	80	56	+14	+20	21	21
7	4	63	82	19	+ 4	+25	18	21
8	5	71	90	19	+16	+26	20	21
9	4	57	76	19	+28	+15	25	19
Average	4	64	83	19	+16	+22	21	20
10	5	74	103	56	+ 2	+ 5	16	14
11	3	23	85	62	+44	+16	23	19
12	3	67	93	26	+51	+14	23	16
13	3	44	116	72	+ 7	+ 1	16	11
14	2	150	279	129	− 2	− 7	14	10
15	2	39	168	128	+ 3	− 1	13	14
16	3	76	148	72	+ 4	− 1	15	11

The testicular extracts Nos. 126 and 130 were prepared by the "dry" method previously described [Korenchevsky, Schalit and Graetz, 1932] and dissolved in sesame oil. The extracts Nos. 4, 89 and 92 were prepared by Koch and Gallagher's method [1929] as far as the stage of extraction from hexane solution with 70 % alcohol. Extract 111, prepared by the same method, was purified to the last stage. These last four extracts were emulsified in 1 % gum acacia. All the extracts were sterilised for 30 minutes on three consecutive days in the steamer at 100° and kept in sealed ampoules in the cold store at 0°. No induration or inflammation was in any case produced by the injections but when the extract was dissolved in sesame oil some amount of unabsorbed oil was always found at autopsy in the subcutaneous tissue at the sites of more recent injections.

The influence of the injections in different litters.

These results are summarised in Table II. The experiments on litters 1 to 10 were designed to show this influence. Those on litters 11 to 16 were preliminary experiments for the purpose of testing the strength of a testicular preparation. In these experiments the difference in the response of different litters became evident. In addition to the organs given in Table II the other endocrine organs and the retroperitoneal fat were weighed. These are not included in the Table, since the amount of the testicular preparation injected

Table II. *The influence of the injection of testicular hormone on the weights of the sexual organs and thymus, calculated per 200 g. of body weight, of the rats in each group of each litter.*

Extract injected and length of period of injections	No. of litter	Prostate and seminal vesicles (mg.) Control rats	Injected rats	Increase %	Penis (mg.) Control rats	Injected rats	Increase %	Thymus (mg.) Control rats	Injected rats	Decrease %
No. 126	1	137	206	50·4	85	110	29·4	382	341	10·7
20 mg.	2	160	280	75·0	88	114	29·6	339	328	3·2
10 days	3	150	253	68·7	100	118	18·0	471	384	18·4
	4	153	274	79·1	97	124	27·8	467	441	5·6
	Average	150	253	68·3	93	117	26·2	415	374	9·5
No. 130	5	60	145	141·6	43	99	130·2	525	415	21·0
12 mg.	6	50	129	158·0	46	95	106·5	464	335	27·8
10 days										
	Average	55	137	149·8	45	97	118·4	495	375	24·4
No. 130	7	157	298	89·8	126	136	7·9	516	325	37·0
12 mg.	8	157	300	91·1	105	129	22·9	359	277	22·8
10 days	9	121	267	120·6	87	108	24·1	460	323	29·8
	Average	145	288	100·5	106	124	18·3	445	308	29·9
No. 130	10	126	221	75·4	104	123	18·3	256	185	27·7
12 mg.										
10 days										
No. 4	11	46	114	147·8	—	—	—	—	—	—
20 mg.	12	184	260	41·3	—	—	—	—	—	—
10 days										
No. 89	13	65	146	124·6	—	—	—	—	—	—
20 mg.	14	199	291	46·2	—	—	—	—	—	—
7 days										
No. 92	15	50	100	100·0	—	—	—	—	—	—
20 mg.	16	110	171	55·5	—	—	—	—	—	—
6 days										

and the length of the period of injections were not sufficient to produce marked changes. During these experiments dissection and investigation of the changes in the thymus were begun. It was found that, like the sexual organs, the thymus is also sensitive to the small amounts of testicular preparations used for the test.

Experiments with testicular extract 126.

The same amount of extract 126 (20 mg. per day per rat) was injected daily for 10 days into two rats of each of the litters Nos. 1 to 4. The remaining litter-mates served as the controls. As can be seen from Table I, the rats of all the litters were castrated at the age of sexual maturity; the injections were made for 10 days and the rats killed 20 days after castration.

The weight of the prostate with seminal vesicles increased in the injected rats, as compared with the controls, on the average by 68·3 %, the deviations from this average not exceeding 26·2 %. The maximum difference in the increases was between the litters 1 and 4, being about 57 % greater in the latter litter than in the former.

The response of the penis to the injections was smaller than that of the

prostate and seminal vesicles, the increase being on the average 26·2 %, with a maximum deviation from the average of 31·3 %.

The response of the thymus was still less, its reduction averaging 9·5 %, with a maximum deviation of 93·6 %.

Experiments with extract 130.

Three groups of litters were injected with extract 130, using 12 mg. per rat daily for 10 days. Litters 5 and 6 belonged to the first group of rats castrated before sexual maturity at the age of 23 and 25 days. Litters 7, 8 and 9 formed the second group of rats castrated after maturity and injected 19 days after castration. The third group, containing only litter 10, was also operated on after maturity, but the injections were made much later, namely, 56 days after castration.

The increase in the prostate and seminal vesicles on injection was much greater in the first group (litters 5 and 6) than in the second and third groups (litters 7–10), as was also the case with regard to the increase in weight of the penis. The decrease in the thymus was on the average nearly the same in all three groups.

This experiment indicates quite clearly that, with regard to the increase in weight of the sexual organs on injection with the hormone, the reaction is much greater in rats castrated before, than in rats castrated after, sexual maturity.

The results are influenced more by the date of operation in relation to maturity than by the length of time between the operation and the beginning of the injections.

Thus the increases in weight of the prostate with seminal vesicles in litters 7 and 8 are very close to that of litter 10, being 89·8 %, 91·11 % and 75·4 % respectively. The maximum difference between litters 8 and 10 was about 21 %, whilst litter 9, castrated at 57 days, *i.e.* close to the date of maturity (perhaps not even having reached maturity), showed a greater deviation from litters 7 and 8, to which group litter 9 belongs, the weight of prostate with seminal vesicles being 34·3 % greater in litter 9 than in litter 7. The maximum deviation from the average, however, of the prostate with seminal vesicles in the second group was 20 %.

Experiments with extracts Nos. 4, 89 and 92.

Six litters were used for these experiments Nos. 11 to 16. Each of the extracts was injected into two litters, one castrated before and the other after sexual maturity (Table II). Although in some litters there were only two rats, one injected and the other serving as the control, the results are identical with those of litters 5–10, *i.e.* in the rats castrated before maturity the increase in the prostate with seminal vesicles on injection was much greater than in those castrated after maturity.

The injection of different doses into rats of the same litter.

These experiments were performed on three litters, Nos. 5, 7 and 17, and the results are summarised in Table III. The results of the injection of litters

Table III. *Percentage changes in the weights of organs after the injection of different doses of testicular extract into groups of rats belonging to the same litter, the food intake per day per 200 g. of body weight and the change in body weight per 200 g. of initial weight.*

		Prostate and seminal vesicles			Penis			Thymus		
			Rats given large dose			Rats given large dose			Rats given large dose	
No. of litter	No. of rats in litter	Rats given small dose	Observed increase	Expected increase	Rats given small dose	Observed increase	Expected increase	Rats given small dose	Observed decrease	Expected decrease
5	6	+142	+263	+213	+130	+191	+195	−21	−27	−31
7	6	+ 90	+129	+135	+ 8	+ 8	+ 12	−37	−43	−56
17	6	+ 39	+ 76	+ 77	+ 1	+ 19	+ 2	—	—	—

		Paste intake per day per 200 g. body weight			Gain in body weight per 200 g. of initial weight			
No. of litter	No. of rats in litter	Control rats	Rats given small dose	Rats given large dose	Control rats	Rats given small dose	Rats given large dose	Extract and daily doses injected
5	6	21	22	21	+ 7	+14	+25	No. 130 12 mg. 18 mg.
7	6	18	21	22	+ 4	+25	+33	No. 130 12 mg. 18 mg.
17	6	21	18	18	+31	+16	+17	No. 111 4 mg. 8 mg.

5 and 7 with the small dose will also be found in Table II), since this small dose and the test preparation, No. 130, were the same as were used for litters 5 to 10. The larger dose of this extract injected was 18 mg. per day.

Litter 17 was castrated at 65 days old, and injections were started at 81 days old, or 16 days after castration, the period of injection being 12 days. Extract 111 was injected into 2 rats of this litter in the daily dose of 8 mg. per rat, the two remaining rats serving as uninjected controls.

In Table III the first two columns for each organ give the changes % actually observed after the injection of two different doses; an increase in the case of prostate with seminal vesicles, a decrease in the case of thymus. In the third column is given the expected increase or decrease with the larger dose, assuming that the increase in the change would be in direct proportion to the increase in the dose. Thus, in litter 17 (see Table IV) the average weight of the prostate with seminal vesicles of the control rats was 191 mg. After the injection of 4 mg. per day per rat the average weight of the prostate with seminal vesicles of the two injected rats was 265 mg., an increase of 74 mg.

Therefore the double dose would be expected to produce an increase of 148 mg. though so close an agreement with the actual result of 146 mg. was probably fortuitous.

In litter 7, however, the expected and actual increases are again very close; though in litter 5 they are more divergent, the actual increase being 263 % while the expected increase was only 213 %.

It is necessary to add that this close agreement is only true for the comparison of the averages of each group. Variations are always present between the individual rats of the group, as was shown in a previous paper [Korenchevsky, 1932, 1].

For instance, in these two litters 7 and 17, giving the striking results mentioned above, the weights of the prostate with seminal vesicles, when calculated per 200 g. of body weight, in the individual rats of the three groups, were as follows:

Table IV.

Weights in mg.

		Litter 7	Litter 17
Two rats of control group		165	193
		149	189
	Average	157	191
Two rats injected with small dose of hormone		314	280
		281	250
	Average	298	265
Two rats injected with large dose of hormone		409	298
		308	376
	Average	359	337

This shows the importance of using at least two rats for a group, in which case the reliability of the average obtained from the data given even by only two rats, as has already been shown in a previous paper [Korenchevsky, 1932, 1], is usually satisfactory, provided that more than one litter is used for the conclusions.

Discussion.

Although the number of experiments performed was not large, very similar results were obtained in all cases. At the same time the corroboration of the results by a large number of experiments on the stocks of rats of different laboratories is necessary.

Some facts, however, became evident. Thus, in castrated rats the prostate and seminal vesicles, weighed together, are the most sensitive organs for the assay of testicular hormone.

The response of these organs is much greater, *i.e.* they are much more sensitive, if the rats are castrated before than after sexual maturity. It seems advisable, therefore, to use rats castrated before sexual maturity for the assay.

Since the exact date of maturity is uncertain it is necessary to castrate the rats before the 30th day after birth and to use only such castrated rats for the tests.

The data are insufficient to enable us to decide what time should elapse after castration before the rats are used for assay.

As has been stated before, however [Korenchevsky, 1932, 2], from the beginning of the second month to 6 months after castration, when this was performed before maturity, the weights of the atrophied prostate and seminal vesicles, calculated for the same unit of body weight, were found to be practically constant. For the other organs, however, more nearly constant results are not obtained until $2\frac{1}{2}$ months after castration. Should it be found that rats, within this period after castration, respond to the same degree to the injections, this period would perhaps be most suitable. In those experiments in which injections of different doses of testicular hormone were made, on the average a remarkably exact correlation was noted, in the form of a direct proportion between the increase of the dose and in the increase in the weight of the atrophied prostate with seminal vesicles. If this is found to be corroborated by more numerous experiments, it will be possible to calculate sufficiently nearly, from any dose injected in a preliminary experiment, the dose necessary for the assay injections. In this case the standardisation of the hormone will be easier than by the comb-growth method, in which only small doses give reliable comparable results.

It seems at the present moment, on the basis of the experiments described in this and the papers mentioned above, that a method of assay on rats belonging to different litters and a rat unit of testicular hormone can be suggested. The following definition is proposed only as a foundation for testing the method experimentally for simplicity, reliability and degree of exactness and for comparison with other methods.

As a rat unit, we suggest the minimum daily dose of testicular hormone injected during 7 consecutive days into at least 3 litters of rats castrated before the 30th day after birth, which will produce, on the average, an increase of 40 % in the weight of the prostate with seminal vesicles as compared with the weight of these organs in the uninjected litter mates.

For the assay the emulsification (or solution if possible) of testicular hormone in watery solvents will be preferable, since when the testicular hormone is injected dissolved in oil uncertain amounts of unabsorbed oil were always found in the subcutaneous tissue at the places of more recent injections. This probably indicates that some of the hormone injected during the assay was not absorbed. The amount of this unabsorbed hormone in oil may vary and become a source of error. In our experiments an attempt to decrease this error was made by increasing the period of injections to 10 days, thus allowing time for the complete absorption of the extract injected during approximately the first 7 days, *i.e.* the greater part of the injected material.

It is clear from Table II that although the increase in weight of the penis and decrease in weight of the thymus confirm the activity of the extract, and therefore, it is advisable to weigh them also, the response is not sensitive and the variations observed are sometimes large as compared with those of the

prostate with seminal vesicles. They are not, therefore, suitable for a quantitative assay.

In Tables I and III the figures showing the increase in weight and the appetite of the injected as compared with the uninjected rats are given. Marked simultaneous decreases of these in the injected rats usually indicate the presence of depressing substances in the preparation. This was noted for instance in litters 11 to 17, which were injected with the first extracts we prepared.

Loss in weight without a diminished, or even with an improved, appetite may indicate an increased metabolism.

In litters 1, 5, 6, 7 and 8 (Tables I and III) better growth was obtained after the testicular injections, especially when larger doses were injected (Table III).

A large reduction in the appetite of the injected rats also checks the normal rate of increase of the weight of prostate and seminal vesicles.

Summary.

1. Using 67 rats belonging to 17 litters a study has been made of the response of rats belonging to different litters to the same dose of testicular extract, and also of the response of rats to different doses of the same testicular preparation.

2. The conditions necessary for the assay of testicular hormone suggested in previous papers were corroborated.

3. In castrated rats the most sensitive organs suitable for the assay of testicular hormone are the prostate with seminal vesicles.

4. Although the penis and thymus respond to the injections simultaneously with the prostate and seminal vesicles, the response is less in degree, is more variable and is less sensitive.

5. The increase on injection in the weight of the atrophied prostate with seminal vesicles is much greater in rats castrated before, than in rats castrated after, sexual maturity.

6. In rats belonging to different litters, injected at a similar period after castration with the same dose of the same testicular extract, the variations in the increase in weight of the prostate with seminal vesicles were not large provided the litters were operated on at the same time in relation to the date of sexual maturity, *i.e.* before or after maturity.

7. In three experiments a very close correlation of direct proportion between the increase of the dose of the injected hormone and the degree of the average increase of prostate with seminal vesicles was obtained.

8. On the basis of the results mentioned in this and other papers a suggestion has been made of a possible method of assay of testicular hormone preparations on rats belonging to different litters.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due.

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CLVII. THE ABSORPTION OF NITROGEN AND OF FAT FROM THE ALIMENTARY CANAL OF RATS KEPT ON A VITAMIN A-DEFICIENT DIET.

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The factors responsible for the pathological condition leading to failure of normal growth in rats kept on a vitamin A-deficient diet are not exactly known. Since the appetite of such rats is less than that of normal rats, the most obvious explanation of the decreased rate of growth would be an insufficient food intake. A second possible cause of the disturbance of rate of growth might be a decreased absorption of food from the alimentary canal. This latter is suggested by the fact that frequently gastro-intestinal disturbances are associated with this dietetic defect. In this paper a preliminary investigation of these two suggestions is described.

Technique.

A litter of four male rats was used for the experiment. Two of the rats were placed on a vitamin A-deficient paste diet and two on the same paste supplemented by cod-liver oil, given by pipette. In addition to the paste, the vitamin A-deficient rats were given as many milligrams of cotton-seed oil as the control rats received of cod-liver oil.

The rats were placed on the experimental diet at the age of 33 days and kept in the usual cages for 24 days, by which time the rate of growth of the rats on the deficient diet had begun to decrease. At the age of 58 days, on the 25th day of feeding with the experimental diet, the rats were placed in metabolism cages in a special room kept at a constant temperature, and all the necessary precautions for accurate metabolism estimations were taken, as described in a previous paper [Korenchevsky and Dennison, 1932]. The paired feeding method was used in these experiments. The rats on the vitamin A-deficient diet, Nos. 1756 and 1757, however, were allowed to consume the paste *ad libitum* since the main object of the experiment was an estimation of the appetite and the absorption of the food. The control rats, Nos. 1758 and 1759, received the same quantity of food, the amount given being equal to the amount eaten on the previous day by rats 1756 and 1757 respectively.

Although the daily food intake was not kept constant some conclusions on nitrogen metabolism can be made owing to the fact that the rats were paired and that the food intake per 200 g. of body weight of the controls was little less than that of the rats on the vitamin A-deficient diet.

In order to avoid decreasing the amount of food given to the control rats on complete diet to such an extent as to be injurious to their condition, each pair was killed on the day that the vitamin A-deficient rat of the pair showed considerable loss of appetite. As can be seen from the composition of the paste, given below, the content of vitamin B in the form of dried yeast was very high. On such a vitamin A-deficient diet a considerable loss of appetite occurs only a few days before death. By observing these precautions the control rats were kept in a healthy condition throughout the experiment, in spite of the adjustment of the consumption of the diet to that eaten by the vitamin A-deficient rats.

The paste, as in previous metabolism experiments, was given in a semi-liquid form in order to prevent the rats from dropping the food inside the cage, and so into the urine and faeces containers. The composition of the paste was as follows:

	Parts by wt.
Heated caseinogen	18
Wheat starch	50
Cotton-seed oil (irradiated ½ hr.)	15
Salt mixture	5
Dried yeast	18·3
Lemon juice	5
Water	181·7

The cotton-seed oil in the amount used in the diet was considered to be a sufficient source of vitamin E [Evans and Burr, 1927]. The control rats always ate their ration completely.

For the estimation of the general absorption from the alimentary canal, the absorption of nitrogenous substances (*i.e.* mainly proteins) and of fat was studied, since the digestion and absorption of these substances are more complicated processes than in the case of carbohydrates or salts and are, therefore, a clearer indication of the state of absorption in general. The estimation of nitrogen in the food, food residues, urine and faeces was made by the Kjeldahl method, whilst for the estimation of fat the Holt, Courtney and Fales [1919] method was used. In this method the soaps of the dried material are first hydrolysed by aqueous HCl, and to this acid mixture is added an equal volume of 96 % alcohol. This is then extracted with ethyl ether and light petroleum in the cold in a special apparatus. In our experience this method of fat estimation gives satisfactory results and we wish to express our thanks to Dr G. A. Harrison for being so kind as to demonstrate the method to us.

Four day periods were chosen for nitrogen and fat estimations, such periods

being sufficiently long to overcome the difficulty of accurate separation of the urine and faeces of each period [Korenchevsky and Dennison, 1932]. However, in order to economise space, the results of the experiments (summarised in Tables I, II and III) are given as averages for 8 day periods, except in the last period, in which, because of the considerable loss of appetite, the periods for the two pairs of rats were 2 and 3 days respectively. At the end of this period the rats were killed, the pair, Nos. 1756 and 1758 on the 84th day of age and the pair, Nos. 1757 and 1759, on the 85th day of age, having been on the metabolism experiment 26 and 27 days respectively.

The influence of a vitamin A-deficient diet on the appetite and weight of the rats.

In Table I the figures in the columns "N intake" and "fat intake" show a slow decrease in the appetite of rats 1756 and 1757 kept on a vitamin A-deficient diet. A very considerable reduction was noted on the last day of the last period (IV), thus decreasing the figure for this period, which, although it lasted only 2 or 3 days, was calculated, for the sake of comparison, for a week. In the first three periods the control rats, Nos. 1758 and 1759, kept on a complete diet, received per 200 g. of body weight less food than those rats with which they were paired, *viz.* 1756 and 1757 respectively, as shown by the nitrogen and fat intake (Table I).

Table I. *The absorption of nitrogen and of fat from the alimentary canal of the paired rats 1756 and 1758, 1757 and 1759 per week, per 200 g. of body weight.*

No. of rat	No. of period	Average wt. of rat g.	Intake N g.	Intake Fat g.	Urine N g.	Faeces Wt. g.	Faeces N g.	Faeces Fat g.	N balance g.	Fat absorbed g.
1756 on	I	140	3·69	15·17	2·51	5·02	0·33	0·81	+0·85	14·36
−A diet	II	150	3·33	13·66	2·42	5·19	0·34	0·83	+0·57	12·83
	III	157	3·00	12·31	2·44	4·42	0·30	0·73	+0·26	11·57
	IV	150	1·36	5·59	1·73	2·88	0·20	0·56	−0·57	5·03
1758 on	I	170	3·34	13·71	2·12	4·85	0·30	0·73	+0·92	12·98
complete	II	191	2·79	11·47	1·81	4·01	0·27	0·70	+0·71	10·77
diet	III	212	2·54	10·44	1·80	3·33	0·23	0·53	+0·51	9·91
	IV	216	1·75	7·20	1·41	2·98	0·19	0·53	+0·15	6·67
1757 on	I	152	3·88	15·93	2·54	5·72	0·37	0·79	+0·97	15·14
−A diet	II	162	3·03	12·45	2·36	4·49	0·29	0·74	+0·38	11·71
	III	161	2·90	11·90	2·33	3·85	0·27	0·64	+0·33	11·26
	IV	157	2·19	9·00	2·14	4·06	0·28	0·74	−0·23	8·26
1759 on	I	163	3·84	15·79	2·22	6·18	0·40	0·87	+1·22	14·92
complete	II	185	2·89	11·85	2·01	4·34	0·31	0·71	+0·57	11·14
diet	III	195	2·63	10·79	1·89	3·35	0·24	0·59	+0·50	10·20
	IV	204	2·49	10·23	1·87	4·08	0·28	0·72	+0·34	9·51

Table III shows the changes in weight compared with the food intake from the beginning of the experiment to the end of the period III, *i.e.* during the first 24 days of the metabolism experiment when the appetite, although less, was still satisfactory. The figures for the last period IV are not included in this Table since in this period the difference in the amounts of food consumed by the deficient and normal rats was considerable. The figures in Table III

are the actual figures, and not the figures calculated per 200 g. of body weight as in Tables I and II.

Table II. *Percentage of the nitrogen intake excreted in the urine, deposited in, or lost from, the body and non-absorbed from the alimentary canal; also percentage of the fat intake non-absorbed.*

No. of rat	No. of period	% N faeces to N intake	% N urine to N intake	% N balance to N intake	% fat non-absorbed to fat intake
1756 on −A diet	I	8·94	67·9	+23·0	5·33
	II	10·21	72·7	+17·1	6·07
	III	10·00	81·3	+ 8·7	5·93
	IV	14·70	127·2	−41·9	10·02
1758 on complete diet	I	8·98	63·5	+27·5	5·32
	II	9·67	64·8	+25·4	6·10
	III	9·05	70·9	+20·1	5·08
	IV	10·85	80·6	+ 8·6	7·36
1757 on −A diet	I	9·53	65·4	+25·0	4·96
	II	9·56	77·9	+12·5	5·94
	III	9·30	80·3	+10·3	5·37
	IV	12·78	97·7	−10·5	8·22
1759 on complete diet	I	10·41	57·8	+31·8	5·50
	II	10·72	69·5	+19·7	5·90
	III	9·12	71·8	+19·0	5·47
	IV	11·24	75·1	+13·6	7·13

Since the control rats were growing more quickly than the vitamin A-deficient rats the amount of food per 200 g. of body weight given to and completely consumed by the rats on a complete diet was less than that consumed by the rats on the vitamin A-deficient diet.

It should also be noted that it was only possible to make an approximate, immediate, estimation of the food eaten on the previous day by the vitamin A-deficient rats, since this was calculated by subtracting the weight of the residue of the food left by the rat from the weight of food given. There is, however, always during the 24 hours, some evaporation of water from the paste left unconsumed.

In later experiments, which will be described in the next paper, the error due to this evaporation is, as far as possible, corrected. For our conclusions, however, it was fortunate that the control rats on a complete diet were allowed less food per 200 g. of body weight than was consumed by the rats on the vitamin A-deficient diet, and in spite of this grew much better. This is especially evident with the pair of rats Nos. 1757 and 1759, for while the rat on deficient diet stopped growing during the 16 days of periods II and III (Table I) and finally lost weight, rat 1759 continued to grow until it was killed. Certainly there is little doubt that the control rats would gain in weight more if the amount of food given were increased.

The rats kept on the vitamin A-deficient diet, however, do not seem to be able to increase in weight in normal correlation to the amount of food ingested. If, from the actual increase in body weight and the actual nitrogen intake of the four experimental rats the increase in body weight per g. of nitrogen intake is calculated, the above statement would seem to be corro-

borated (Table III). Thus, whilst 1 unit of vitamin A-deficient food, as measured by 1 g. of nitrogen, produced an increase in body weight of only about 2·5 g., the same amount of complete diet produced an increase in the weight of the two control rats of 6·54 and 5·55 g. respectively, *i.e.* the increase in weight in the control rats was on an average 143 % greater than in the deficient rats.

Table III. *Gain in body weight per g. of nitrogen intake during the experimental period of the first 24 days.*

No. of rat	Total gain in weight (actual) g.	N intake (actual) g.	Gain in weight per g. of N intake g.
1756 –A	21	8·47	2·48
1758 Nor.	61	9·31	6·54
1757 –A	22	8·80	2·50
1769 Nor.	53	9·54	5·55

From the above experiments it is obvious that:

(1) the body weight of rats kept on vitamin A-deficient diet did not increase in normal correlation to the amount of food ingested;

(2) during the periods when the growth of the rats is checked or remains stationary the decrease in the appetite, with the consequent decrease in food intake, of rats kept on a vitamin A-deficient diet could only partly explain the decrease in rate of growth, and as a chief cause is probably only applicable to the last stages of the deficiency.

Nitrogen and fat absorption.

The comparison of the figures for nitrogen and fat excreted in the faeces, *i.e.* non-absorbed, shows that absorption during all the periods except the last was slightly less in only one of the rats on the vitamin A-deficient diet, No. 1756, as compared with its paired control, No. 1758 (Table I). Only in the last period was the absorption considerably affected in both deficient rats. When absorption of nitrogen and fat is expressed as the percentage of the intake (Table II) it is evident that such a small decrease in the absorption cannot explain the difference in growth.

This conclusion is seen to be justified in view of the results obtained with the second pair of rats Nos. 1757 and 1759, there being no constant appreciable difference in absorption in these two rats during the first three periods (Tables I and II), whilst the difference in growth still occurred.

It is true that during the last (fourth) period (just before the rats were killed) the percentage of non-absorbed nitrogen and fat (Tables I and II) was more pronounced in the deficient than in the control rats. It must be mentioned, however, that in rat 1756 there was an inclination to diarrhoea, and only in this rat was the non-absorbed nitrogen raised to 14·7 % and the non-absorbed fat to 10·02 %. During this last period, close to death, the absorption of nitrogen and fat were decreased more noticeably but still not very greatly.

Taking all the above data into consideration the following statements can be made.

(1) Except for a period of a few days before being killed, the absorption of nitrogenous and fatty substances from the alimentary canal was very slightly, if at all, decreased in rats kept on the vitamin A-deficient diet.

(2) Therefore the checking or cessation of growth of rats on a vitamin A-deficient diet cannot be explained by a decrease in the function of absorption of food from the alimentary canal.

(3) Only during the last period before death, when the vitamin A-deficient rats have begun to lose weight, and also to have an inclination to diarrhoea, does any impairment of the function of absorption become apparent and even then not to a very great extent.

Nitrogen metabolism.

Since the rats were young, nitrogen deposition for growth should be considerable. In both the vitamin A-deficient rats, as compared with their paired controls, although the nitrogen intake per 200 g. of body weight was greater, the deposition of nitrogen in the body was less (Table I). The reduction in food intake also produced a greater reduction in the nitrogen deposition in the deficient rats than in the rats on the complete diet. Table II shows also that in the vitamin A-deficient rats the percentage of the nitrogen intake excreted in the urine was greater, while that deposited in the body was less than in their respective controls.

These facts indicate that from the beginning of the metabolism experiment, namely, from the 25th day of feeding with the vitamin A-deficient diet to the end of the experiment (*i.e.* for about 4 weeks), the nitrogen metabolism of these rats was increased, during some periods to a marked degree (compare the periods II, III and IV in Tables Nos. I and II).

Discussion.

From this observation on the loss of appetite and consequent decrease in food intake, and also in some instances a lessened absorption from the alimentary canal in rats kept on a vitamin A-deficient diet, it is possible to conclude that these factors alone cannot explain completely the checking of growth which was observed. In rats kept on a vitamin A-deficient diet, during the period preceding death, when the loss of appetite becomes considerable, the influence of the decreased food intake on the rate of growth seems to become the important, and probably, the chief factor. Even in this period, however, and more particularly in the previous periods, the increased nitrogenous metabolism (*i.e.* chiefly protein metabolism), may be one of the factors which check the growth of the vitamin A-deficient rats, since the deposition of nitrogen has already been decreased by a lessened food intake. Certainly with a normal appetite such a degree of increase of metabolism as was observed in the experiment would hardly alone be able to check growth. This patho-

logical condition of metabolism may be the direct result of deficiency of vitamin A, or the indirect result of the deficiency on the endocrine organs controlling metabolism. The data at present available give no indication as to which of these factors is responsible.

The chief factor in checking the growth seems to be that one unit of the vitamin A-deficient food consumed by the rats produces a much smaller increase in body weight than the same unit of complete diet. Perhaps this fact is correlated with the increased metabolism. If even a slight pathological condition of absorption is also present the total effect on growth will be greater.

Summary.

1. The body weight, appetite, intake and absorption of fat and nitrogen, and the nitrogenous metabolism of two male rats kept on a vitamin A-deficient diet were compared with those of two control litter-mates kept on a complete diet.

2. Each control rat was paired as regards food intake and the other conditions of the experiment with a rat on a vitamin A-deficient diet.

3. During the period of checking or cessation of growth of the rats the decrease in food intake was only moderate and could offer only a partial explanation of the change in rate of growth, since the amount of food, if calculated per 200 g. of body weight, consumed by the rats on the deficient diet was even larger than that consumed by the rats on the complete diet.

4. During the same period the absorption of nitrogen and of fat was not appreciably altered and could not, therefore, be the factor responsible for this checking of growth.

5. In the last period, when the appetite was greatly decreased, the decrease in food intake was probably the main cause of the loss of weight, this effect being augmented by the definitely decreased absorption from the alimentary canal observed during the last period.

6. During the whole period of observation the nitrogenous metabolism increased.

7. This increase of metabolism occurring simultaneously with the decreased food intake may check the normal growth of rats kept on a vitamin A-deficient diet.

8. In such rats the increase in body weight was less than in rats kept on a complete diet when correlated with the same unit of food ingested. This fact would seem to be the chief factor in checking the growth of rats kept on a vitamin A-deficient diet.

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CLVIII. THE INFLUENCE OF VITAMIN A DEFICIENCY ON MALE RATS IN PAIRED FEEDING EXPERIMENTS.

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The object of this investigation was to determine whether the changes produced in body weight and in the deposition of fat in male rats on vitamin A-deficient diets are due to this deficiency alone or to the accompanying reduced appetite; and also to study the changes in weight of the sexual and endocrine organs.

The following experiments, therefore, supplement those published in a previous preliminary paper [Sampson, Dennison and Korenchevsky, 1932].

In the preliminary paper observations were made on 2 rats on vitamin A-deficient diet and on 2 control rats on a complete diet, paired in regard to food intake and other conditions with the respective rats on the vitamin A-deficient diet.

In spite of the decreased appetite during the period of cessation of growth the rats on vitamin A-deficient diet (– A diet) consumed a larger amount of food per 200 g. of body weight than did those on the complete diet. At the same time one unit of the vitamin A-deficient food consumed by the rats produced a much smaller increase in body weight than the same unit of complete diet. The latter fact was perhaps correlated with the simultaneously increased nitrogen metabolism which was observed. It was suggested that these facts give the chief explanation of the checking of growth produced by the direct deficiency of vitamin A irrespective of appetite since, during this period, the absorption from the alimentary canal did not show any significant changes.

We were unable to find in the literature any record of the use of the paired-feeding method in investigations of vitamin A deficiency, or, consequently, any accurate estimations by weight of changes in fat deposition, or in sexual or endocrine organs due directly to vitamin A deficiency.

It is known that, in general, fat deposition is decreased in animals kept on a vitamin A-deficient diet. It is also known that, in such animals, the actual weight of the testes is less than in those on unlimited complete diet. Histological examination reveals degeneration of the seminiferous tissue [see references by Mason, 1930].

A critical review of these references will be given in a subsequent paper elsewhere, when dealing with the histological changes observed in the sexual and endocrine organs of the rats on which the experiments described in this paper were performed.

Technique.

The experiments were performed on 63 male rats belonging to 12 litters. They were reared on the stock diet used at the Lister Institute, described by Smith and Chick [1926]. The litters were placed on the experimental diets at the following ages:

Litters 6 and 7 on the 7th day.
Litters 1, 2, 3, 5, 10, 11 and 12 on the 21st to 26th day.
Litters 4, 8, 9 on the 27th to 33rd day.

The duration of the experiment was:

Litter 8—24 days.
Litters 1, 2, 3, 6, 7 (except rats 30 and 32, 28 days)—9 days.
Litters 10, 12—31 to 35 days.
Litters 5, 11—39 to 42 days.
Litter 4—52 days.

The final ages and the weights of the rats are given in Table III.

In Exps. I and II, the diet was given *ad libitum* to all the rats. In the remaining ten experiments the rats were paired. One member of each pair received a diet deficient in vitamin A only, the other member a diet adequate in all respects. On the vitamin A-deficient diet the food intake gradually decreases. This may affect not only the body weight but also the weight of the organs and their structure and function. To discriminate between the effect of decreased food intake and that of the vitamin A deficiency, it is necessary that the paired control animal should receive the adequate diet in the same quantity as was consumed by the litter mate on the deficient diet. This procedure, adopted in this and the previous investigations [Sampson *et al.*, 1932] did not necessitate any considerable reduction in the food intake of the control rats since the pairs were killed on the day on which the vitamin A-deficient litter-mate showed a marked loss of appetite, which sometimes also resulted in a loss of body weight. Owing to these precautions the paired control rats were kept in good health throughout the whole experiment. In order to make a comparison, in some experiments a third litter-mate was allowed the complete diet *ad libitum*.

The period required for exhaustion of the vitamin A reserves in the young rats may be shortened by substituting a vitamin A-deficient diet for the usual diet at the 17th day of lactation—the time when the young begin to consume the stock food directly. Of course, the mother is also consuming this deficient diet from this period. By this method, as was shown by Korenchevsky [1922], the effects of the deficiency may be accentuated. In an endeavour to obtain

extreme changes the mothers and the young of the litters Nos. 6 and 7 were placed on a vitamin A-deficient diet from the 17th day of lactation.

The composition of the – A diet was as follows: caseinogen (heated) 180, starch (wheat) 500, cotton-seed oil (irradiated) 150, salt mixture 50, yeast (dried) 183, lemon juice 50, water 700 parts by weight.

The control animals received the same diet supplemented with daily doses of 20 to 100 mg. (according to the weight of the animal) of cod-liver oil (Fairbank Kirby). To keep the caloric intake of the paired litter-mates equal, the rats on vitamin A-deficient diet received an equal amount of hydrogenated cotton-seed oil.

In Exps. I and II, however, the control animals received a paste in which the cotton-seed oil was replaced by 120 parts of butter and 30 parts of cod-liver oil. All the rats of these two litters also received 6 drops of wheat-germ oil daily to supply an extra amount of vitamin E and a small though inadequate amount of vitamin A, so that the vitamin A deficiency in litters 1 and 2 was much less complete than in litters 3 to 12. Vitamin E was supplied in the latter experiments by hydrogenated cotton-seed oil, since according to Evans and Burr [1927] this fat, when fed in the amount given above, is a satisfactory and sufficient source of the antisterility vitamin.

The diet in all the experiments, except IX, was given in the form of a paste. In Exp. IX the rats were given a diet of the same composition but without the addition of water. It was hoped that a more accurate weighing of the dry residue would thus be obtained. Unfortunately the vitamin A-deficient rats during the period of pronounced deficiency scattered this dry diet badly and the collection of the residue was much more difficult than with the paste.

At the same time the accurate estimation of food eaten by the deficient rats was of the utmost importance since this amount determined the amount of diet to be given to the paired control on the following day.

The chief difficulty was to determine the amount of the residue of the paste since, by the end of 24 hours when the residue was collected, some of the water in the paste had evaporated.

In order to prevent this evaporation as much as possible and also to prevent the scattering of the food the paste was firmly pressed into heavy metal cups. Though helpful, this precaution was insufficient, so that it was necessary to introduce a correction for the evaporation of water.

The amount of evaporation of water from the diet was determined in the following way. Weighed samples of the paste in quantities equal to the amount of food given to the rats and to the amount of residue usually left were placed on the same shelves as those on which the rat-cages were kept. At the end of 24 hours the samples were reweighed and the amount of water evaporated estimated. At frequent periods during the investigation observations of the evaporation were made and on the average the loss of water was found to be nearly constant, probably owing to the fact that the room temperature was kept practically constant.

Table I. *Average evaporation of water from the food residue at the end of 24 hours.*

Weight of sample g.	Loss of water by evaporation g.
1 to 2	0·5
2 to 5	1·0
5 to 10	2·0

The loss of water was on the average as given in Table I.

These figures indicate that to the weight of paste uneaten at the end of 24 hours and partially dried, from 0·5 to 2 g. must be added according to the weight of the residue to offset the evaporation of water.

In order to estimate the accuracy of this method of correction for evaporation the data of the food consumption obtained in this way were compared with the food consumption calculated on the basis of the nitrogen intake in the experiment described in the previous paper [Sampson, Dennison and Korenchevsky, 1932]. The results are summarised in Table II.

Table II. *The relation of the quantity of paste consumed, estimated by weighing the paste and residue and correcting for evaporation, to 1 g. of the actual intake of nitrogen, for the different periods of the experiments on rats 1756 and 1757.*

Period		Corrected paste intake	Nitrogen intake by analysis	Relation of paste intake to 1 g. N intake
1756	I	127	2·70	47·0
	II	113	2·46	45·9
	III	110	2·45	44·9
	IV	122	2·52	48·4
	V	120	2·51	47·8
	VI	105	2·19	47·9
	VII	46	1·02	45·1
1757	I	138	2·93	47·1
	II	137	2·93	46·7
	III	116	2·51	46·2
	IV	114	2·38	47·9
	V	100	2·12	47·2
	VI	120	2·54	47·2
	VII	78	1·72	45·3
			Average	46·7

If the method were perfect 1 g. of nitrogen would always have corresponded exactly to the same amount of paste, for which the nitrogen content was found, by analysis, to have been constant throughout the experiment. Fluctuations, however, were observed, the lowest figure being 44·9 and the highest 48·4, with an average of 46·7. Since the maximum deviation from the average did not exceed 3·9 % this method of correction may be considered to be quite satisfactory.

To the rats mentioned in Table II the paste was given in a semi-liquid form, while to all other rats it was given in the form of a dough-like paste. In these latter cases, therefore, some scattering of the food always occurred and consequently the possible error was greater. Great care was taken,

however, to collect accurately the scattered food and the scattering was considerably reduced by pressing the ration firmly into the metal cups to which it adhered quite well.

For the interpretation of the changes in the thyroid the consideration of the amount of iodine present in the diet is of importance. The vitamin A-deficient diet used differed in iodine content from the complete diet since the cod-liver oil given to the rats on the complete diet contains iodine, while cotton-seed oil does not. Therefore some of the rats of litters 10, 11 and 12 (marked with an asterisk, Table III) were given 7 and 30 γ of iodine per day dissolved in cotton-seed oil. Both these doses, in any case, are larger than the maximum amount of iodine in the daily quantity of cod-liver oil given to the rats [see Orr and Leitch, 1929, p. 88].

All rats were weighed at least twice weekly, and examined for symptoms of vitamin A depletion. At the onset of xerophthalmia the rats were weighed daily. The experiments were continued for 24 to 52 days in each case until xerophthalmia was obvious or until loss of appetite led to slower gain in, or loss of, body weight. The rats were killed by coal gas, pairs being killed simultaneously.

For dissecting and weighing the organs the technique proposed by Korenchevsky [1930; 1932] was used. Thus the intra-abdominal fat and penis were weighed fresh, while the testes, prostate with seminal vesicles, thyroids, adrenals, pituitary and thymus glands were weighed after remaining in Allen's modification of Bouin's fixative for 48 hours. The fixed material was subsequently used for histological study.

Experimental results.

Body weight and appetite.

The age and the initial and final weights of the rats are given in Table III. The abbreviations for the diets which are employed in the tables are also used in the text (see footnote to Table III). Since the −A diet used in the experiments contains a large amount of vitamin B in the form of dried yeast, the rats on this diet suddenly lose their appetite, usually only a few days before death. As mentioned above, on the day when a considerable loss of appetite and of body weight occurred the pairs were killed. The gain or loss in weight during the last day is given, to indicate the condition, *i.e.* retarded growth, cessation of growth or fall in weight at this time.

It should be noted that of the 34 rats on the − A diet, loss of weight, generally small, occurred in 19 rats only, whilst xerophthalmia was noted in all except the rats of litter 1. The initial weights of the rats of this litter on −A diet were greater than those of any other rats on this diet, and the gain in weight was continuous up to the day of killing. This shows that at killing vitamin A reserves were still present in these rats.

Table III. *Body weight of rats in g.*

	CoU diet			CoP diet			− A diet				
		Weight			Weight			Weight			
Litter no.	Rat no.	Initial	Final	Rat no.	Initial	Final	Rat no.	Initial	Final	Gain or loss during last day	Final age in days
1	1	58	289	—	—	—	3	56	259	+ 4	62
	2	58	289	—	—	—	4	61	257	+ 3	62
2	5	40	231	—	—	—	8	38	185	− 5	58
	6	36	254	—	—	—	9	40	179	+ 3	58
	7	35	237	—	—	—	10	38	180	− 5	58
3	—	—	—	11	30	146	13	34	119	− 7	56
	—	—	—	12	31	147	14	35	130	+ 3	56
	—	—	—	—	—	—	15	33	119	+ 3	56
4	—	—	—	16	48	214	18	50	145	− 9	84
	—	—	—	17	48	205	19	48	149	−16	85
5	20	46	249	22	45	251	24	45	195	+ 3	68
	21	38	264	23	40	222	25	41	149	− 8	68
	—	—	—	—	—	—	26	43	180	− 3	68
6	—	—	—	27	38	123	28	38	121	− 1	55
7	—	—	—	29	38	150	31	40	130	+ 1	55
	—	—	—	30	35	87	32	35	68	− 7	49
	—	—	—	—	—	—	33	36	109	± 0	55
8	34	37	95	—	—	—	37	35	57	−10	51
	—	—	—	35	43	91	38	42	87	± 0	51
	—	—	—	36	35	78	39	39	73	− 4	51
9	40	38	165	42	39	134	44	41	130	− 1	62
	41	39	165	43	41	148	45	43	130	− 4	59
10	—	—	—	46	42	145	49	51	142	+ 1	64
	—	—	—	—	—	—	50	51	115	−10	61
	—	—	—	47	48	152	51*	48	135	− 3	64
	—	—	—	48	44	133	52*	48	112	−11	61
11	—	—	—	53	50	185	55	50	155	− 9	67
	—	—	—	—	—	—	56	47	164	+ 2	67
	—	—	—	54	43	190	57*	52	165	± 0	67
	—	—	—	—	—	—	58*	49	147	− 5	67
12	—	—	—	—	—	—	60	55	123	− 1	63
	—	—	—	—	—	—	61	47	128	+ 2	63
	—	—	—	59	49	148	62*	49	122	+ 2	63
	—	—	—	—	—	—	63*	44	101	+ 3	63

Note. In the tables of this paper the following abbreviations are used: CoU = complete unlimited diet; CoP = complete paired diet, *i.e.* given in the same amount as is consumed by the paired rat on the vitamin A-deficient diet; − A = vitamin A-deficient diet.

In columns CoP and − A the numbers in the same line indicate paired animals.

* These rats received iodine.

Since both in litter 1 and litter 2 rats on the deficient diet received small amounts of vitamin A in the wheat-germ oil, these two litters are considered separately from the other 10 litters in all our averages and interpretations.

The weight and the appetite of the rats must be considered simultaneously. The actual food intake and the intake per 200 g. of body weight are given in Table IV. As can be seen from the figures in this table small discrepancies exist in most pairs between the amounts of food eaten by the rats of a pair. In Table IV in each of the litters in which the paired feeding method was used, *i.e.* litters 3 to 12, the figures of the paired − A-rat are placed below those of its paired control (CoP)-rat, *e.g.* in litter 3 in the column "Actual food

Table IV. *Daily average of food intake in g., actual and per 200 g. of body weight; and average daily gain of each rat per 10 g. of actual food intake.*

Litter no.	Diet	Actual food intake	Intake per 200 g. body weight	Gain per 10 g. of actual food intake
1	CoU	33·1, 30·4 — —	37, 34 — —	1·9, 2·1 — —
	−A	27·1, 25·1 — —	34, 31 — —	2·0, 2·1 — —
2	CoU	26·8, 27·9, 26·0 —	42, 41, 39 —	2·0, 2·2, 2·1 —
	−A	22·3, 20·4, 20·7 —	39, 36, 34 —	1·7, 1·8, 1·8 —
3	CoP	16·8, 16·2 — —	39, 37 — —	2·0, 2·0 — —
	−A	16·0, 16·5, 14·6 —	38, 40, 40 —	1·5, 1·6, 1·7 —
4	CoP	17·6, 17·6 — —	26, 27 — —	1·8, 1·7 — —
	−A	16·3, 17·7 — —	27, 29 — —	1·1, 1·1 — —
5	CoU	24·1, 26·4 — —	35, 39 — —	2·0, 2·0 — —
	CoP	23·7, 21·4 — —	36, 34 — —	2·1, 2·0 — —
	−A	23·6, 20·2, 22·6 —	37, 37, 37 —	1·5, 1·3, 1·4 —
6	CoP	11·5 — — —	32 — — —	2·2 — — —
	−A	16·9 — — —	43 — — —	1·4 — — —
7	CoP	16·3, 10·4 — —	37, 37 — —	2·0, 1·8 — —
	−A	17·0, 10·1, 14·3 —	40, 36, 37 —	1·6, 1·2, 1·5 —
8	CoU	12·6 — — —	41 — — —	1·9 — — —
	CoP	12·1, 11·4 — —	34, 36 — —	1·7, 1·6 — —
	−A	12·2, 11·5, 10·6 —	36, 36, 36 —	1·5, 1·2, 0·9 —
9	CoU	19·2, 20·5 — —	40, 41 — —	1·9, 2·0 — —
	CoP	15·7, 16·6 — —	38, 38 — —	1·8, 2·1 — —
	−A	16·5, 18·3 — —	37, 39 — —	1·6, 1·5 — —
10	CoP	16·0, — 17·2, 16·5	34, — 32, 35	1·8, — 1·7, 1·7
	−A	17·4, 16·6, 17·1, 15·6	35, 32, 32, 33	1·5, 1·2, 1·5, 1·3
11	CoP	19·7, — 19·8, —	32, — 33, —	1·8, — 1·9, —
	−A	20·1, 20·2, 21·0, 18·8	34, 36, 36, 33	1·3, 1·5, 1·4, 1·3
12	CoP	— — 15·0, —	— — 32 —	— — 1·9 —
	−A	15·7, 15·7, 14·7, 12·7	33, 36, 34, 35	1·2, 1·5, 1·4, 1·3

Note. Each figure relates to a rat in the order of the respective numbers of the rats, which can be ascertained by reference to the corresponding litters and groups in Table III.

intake" the figures for the respective pairs are 16·8 and 16·0; 16·2 and 16·5; in the next column they are 39 and 38; 37 and 40 and so on.

When there is a slight excess in the amount of food eaten by the control paired rats as compared with that eaten by the −A rats it is chiefly due to differences in intake on the first and last days of the experiment. On the first day of the experiment the control paired rat may eat more than the deficient rat since actual pairing does not start till the second day when the control rat receives the same amount as was eaten the previous day by the −A rat. On the last day of the experiment, in most cases, the food was completely consumed by the control rats, while the deficient rats might eat very little.

When the amount of food eaten by the control rats is slightly less than that eaten by the −A rats, it is due, in some cases, to giving a little less food to the CoP rats. This was done in order to be certain that the control rats did not receive more than the experimental rats and in order to increase the effect of limitation of the diet. Care was taken, however, not to injure the healthy condition of the control rats by such adjustments. In other cases

the smaller intake of the CoP rats was due to their refusal to consume the diet completely. As is well known some rats dislike synthetic diets. A consideration of these CoP rats on reduced intake as compared with their pairs is of especial importance in our conclusions.

Of those rats receiving the complete unlimited diet, the body weight, in about half of the individuals, was only slightly greater than that of the rats on a complete but paired diet (Table III, litters 5, 8 and 9). This shows that, because of the precautions mentioned above, the limitation of the diet of the paired rats was very small (see Table IV), except in those cases in which the rats disliked the diet and, therefore, left part of it unconsumed.

A comparison of the paired rats reveals that the gain in weight of the rats on CoP diet is greater than that of the rats on −A diet. In a few cases, rats 27, 35, 36 and 42, this difference is small, from 2 to 9 g., but in all the other CoP rats it is much greater, being from 12 to 30 g. in 9 rats and from 31 to 74 g. in the remaining 6 rats. At the same time it can be seen from Table IV that in litters 3 to 12 the food intake of the rats on CoP diet was, in most cases, the same as that of the rats on −A diet or even less. This is evident in the figures for actual food intake but is accentuated when the intake is calculated per 200 g. of body weight. For litters 1 and 2, in which the vitamin A deficiency was only partial, the increase in weight produced by one unit of food complete or deficient in vitamin A was also calculated. The results obtained are given as "Gain per 10 g. of actual food intake," in the third column of Table IV. In all the rats which showed vitamin A deficiency, even in those of litter 2, the gain in weight produced by one unit of vitamin A-deficient diet was, without exception, less than that produced by the same unit of complete diet. In litter 1, in which, as was mentioned above, the depletion of vitamin A was incomplete, this difference in rate of growth per unit of food intake of −A as compared with CoP diet did not occur. The absence of this change in this litter only emphasises its presence in all the other litters. In litters 3 to 12 the actual daily average of food intake for the rats on CoP and deficient diets was 16·4 and 16·6 g. respectively; the average daily food intake per 200 g. of body weight was 34 and 36 g. respectively and the average daily gain in weight per 10 g. of actual food intake 1·9 and 1·4 g. respectively. It is worthy of note that the gain in weight per 10 g. of food intake is nearly the same in the rats on complete diet, in litters 5 and 9, both when consumed *ad libitum* (CoU diet) and when adjusted in quantity to that consumed by the deficient rats. In litter 8 the gain in weight on CoU diet was greater.

In litters 5, 8 and 9, in which all three groups are represented, the body weights in groups CoP and −A are on the average about 11 % and 26 % respectively less than in group CoU. In other words, roughly two-fifths of the deficiency in weight of −A rats in these experiments was due to decreased appetite and three-fifths was due to the direct influence of vitamin A deficiency.

These data also indicate that in order to obtain the same increase in body weight it is not sufficient to ingest the same unit of food. Only if vitamin A is present is this so, otherwise the food ingested in the same quantity will produce a smaller increase in body weight and presumably in the growth of the animal.

Fat deposition.

As judged by the average weights of retroperitoneal and testicular fat in litters 2 to 12, the weight of the fat was less in the −A rats than in those on complete diet (Table V). In the three litters 5, 8 and 9, in which all three

Table V. *Average weight of body fat of rats in each group of each litter (g.).*

	CoU diet			CoP diet			−A diet		
		Per 200 g. body wt.			Per 200 g. body wt.			Per 200 g. body wt.	
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation
1	9·68	6·69	±0·69	—	—	—	7·75	6·01	±0·16
2	6·23	5·34	+0·25 −0·42	—	—	—	2·60	2·87	+0·26 −0·17
3	—	—	—	4·55	6·21	±0·43	2·92	4·77	+0·41 −0·79
4	—	—	—	8·64	8·20	±2·16	3·60	4·88	±1·41
5	9·18	7·17	±0·44	6·01	4·95	±2·02	3·24	3·65	+0·43 −0·85
6	—	—	—	2·96	4·80	±0	2·85	4·70	±0·00
7	—	—	—	1·97	3·11	±0·76	1·42	2·58	+0·82 −1·05
8	1·07	2·25	±0·00	0·77	1·78	±0·53	0·53	1·02	+0·26 −0·28
9	4·87	5·90	±0·09	2·53	3·54	±0·85	2·08	3·19	±0·27
10	—	—	—	3·53	4·91	+1·03 −0·54	1·84	2·94	+0·64 −0·68
11	—	—	—	7·33	7·81	±0·39	4·58	4·47	+1·48 −1·45
12	—	—	—	4·25	5·74	±0	1·84	3·07	+0·41 −0·78

groups are represented, the percentage deficiency of fat per 200 g. of body weight in the CoP group and in the −A group, in relation to the CoU group, and in the −A group in relation to the CoP group, was calculated.

It was found that on the average in these three litters (1) the fat deposition was decreased by about 31 % when the amount of complete diet was adjusted to that consumed by the −A rats; (2) it was decreased by about 26 % in the rats on vitamin A-deficient diet as compared with those which consumed, on an average, the same, or even slightly less, amounts of food rich in vitamin A; (3) the deficiency of fat in the −A group in relation to the CoU group was on the average about 49 %. In litter 2 this decrease was 46 %. Therefore, about three-fifths of the deficiency of fat deposition was due to the decrease in appetite and two-fifths to the direct influence of the vitamin A deficiency. In litters 3 to 12 the percentage deficiency of fat in the

rats in the −A group in relation to those in the CoP group was 23, 40, 26, 2, 17, 21, 40, 40, 33 and 47; on the average 29 %, which is nearly the same as the 26 % obtained with the three litters mentioned above. The unusually small deficiency of 2 % occurred in litter 6, in which the control rat in the CoP group ate much less than his litter-mate in the −A group (see Table IV).

Certainly the decrease of stores of fat in the body will vary with the period of time at which the −A rats are killed. If, however, as was the case in our experiments, the rats are killed in a more or less satisfactory nutritional condition, with xerophthalmia well developed, but a few days before or at the beginning of the marked fall in weight about three-fifths of the average deficiency will be due, at this period of vitamin A deficiency, to the decreased food intake, and about two-fifths of the deficiency to the direct influence of vitamin A deficiency irrespective of the appetite.

Testes.

In general the actual weight of the testes in −A groups was smaller than that of the rats on complete diet. In litters 5, 8 and 9 the weights of the testes per 200 g. of body weight (Table VI) were slightly greater (about

Table VI. *Average weight of testes of rats in each group of each litter (g.).*

	CoU diet			CoP diet			−A diet		
		Per 200 g. body wt.			Per 200 g. body wt.			Per 200 g. body wt.	
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation
1	3·00	2·08	±0·02	—	—	—	2·81	2·18	±0·07
2	2·66	2·21	+0·06 −0·04	—	—	—	2·31	2·55	+0·43 −0·41
3	—	—	—	1·97	2·68	±0·09	1·95	3·13	+0·13 −0·05
4	—	—	—	2·30	2·20	±0·03	1·86	2·53	±0·06
5	2·42	1·89	±0·10	2·55	2·17	±0·19	1·78	2·00	+0·42 −0·50
6	—	—	—	1·69	2·75	±0·00	1·17	1·94	±0·00
7	—	—	—	1·55	2·57	±0·18	1·32	2·90	+0·54 −0·58
8	1·16	2·45	±0·00	1·17	2·79	±0·26	1·05	2·91	+0·20 −0·12
9	1·84	2·23	±0·02	1·73	2·45	±0·01	1·76	2·70	±0·18
10	—	—	—	1·80	2·52	+0·10 −0·13	1·67	2·68	+0·49 −0·33
11	—	—	—	2·54	2·71	±0·10	1·97	2·52	+0·88 −0·70
12	—	—	—	1·88	2·54	±0·00	1·36	2·30	+0·22 −0·34

13 %) in the group CoP than in the CoU group. In the −A group in relation to the same group CoU, the average increase was about the same, namely 15 %.

Although the actual weight of the testes of the rats on vitamin A-deficient diet was always less than that of the rats on CoP diet, when calculated per

200 g. of body weight, the change was not constant. There was an increase of 6 to 17 % in 6 litters, a decrease of 7 to 9 % in 3 litters, of 30 % in litter 6. Taking into consideration the fact that in litters 1 and 2 there was also an increase of 5 % and 15 %, and that, therefore, in most litters the testes of rats on vitamin A-deficient diet weighed more per 200 g. of body weight than those on CoP diet, it must be admitted that, as judged by the weight of the organs, in relation to the same unit of body weight, vitamin A deficiency seems to indicate hypertrophic rather than atrophic changes in the testes. This was not expected since, histologically, in all the testes of the above rats on vitamin A-deficient diet, degenerative or atrophic changes were found. No degeneration was found in the testes of the rats on the paired complete diet. These results will be communicated in detail later. An explanation, however, may be found in the fact that macroscopically these testes appeared to be oedematous, being soft and semi-transparent as compared with the testes of the control rats which were of firm consistency and of milky colour. Oedema of the testes would explain their greater weight.

Penis.

Table VII shows that in all the litters except 1 and 6, the penis, per 200 g. of body weight, was on the average about 21 and 24 % heavier in rats on −A diet than in rats on CoU or CoP diets respectively. In litter 1 there was practically no change, while in litter 6 the penis was smaller in the −A rats.

Table VII. *Average weight of penis of rats in each group of each litter (mg.).*

	CoU diet			CoP diet			−A diet		
		Per 200 g. body wt.			Per 200 g. body wt.			Per 200 g. body wt.	
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation
1	267	185	+25	—	—	—	283	180	± 3
2	219	182	± 1	—	—	—	199	219	+ 9 − 7
3	—	—	—	160	218	± 1	162	263	+48 −45
4	—	—	—	180	172	± 9	191	260	±10
5	224	175	± 4	210	178	±11	184	214	+28 −19
6	—	—	—	142	230	± 0	117	193	± 0
7	—	—	—	98	166	± 1	104	208	+33 −28
8	70	147	± 0	64	151	± 0	67	187	+17 − 9
9	160	194	±24	131	187	±13	143	219	±12
10	—	—	—	160	230	±20	165	260	±20
11	—	—	—	170	180	± 1	175	220	+20 −10
12	—	—	—	150	200	± 0	145	240	+20 −10

Litter 6 was exceptional, owing to the great difference in food intake between the control and −A rats (see Table IV) as was mentioned previously. The influence of vitamin A deficiency on the weight of the penis can be ascribed to the direct action of the deficiency since there was no significant change between the CoU and CoP groups (per 200 g. of body weight).

Prostate with seminal vesicles.

As can be seen from Table VIII decreased food intake caused a decrease in weight of the prostate with seminal vesicles, both in the actual figures and per 200 g. of body weight (compare litters 5, 8 and 9, CoU and CoP groups). Per 200 g. of body weight, on the average, this decrease was about 19 %.

Table VIII. *Average weight of prostate and seminal vesicles of rats in each group of each litter (mg.).*

	CoU diet			CoP diet			−A diet		
		Per 200 g. body wt			Per 200 g. body wt.			Per 200 g. body wt.	
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation
1	1129	782	± 20	—	—	—	1189	922	± 22
2	888	738	+ 7 − 9	—	—	—	583	644	+113 −131
3	—	—	—	434	592	± 24	439	716	+243 −236
4	—	—	—	838	803	±129	887	1206	± 28
5	1257	975	±159	1066	906	± 72	878	1012	+ 99 − 52
6	—	—	—	269	438	± 0	262	434	± 0
7	—	—	—	216	346	± 71	203	388	+ 75 − 51
8	156	328	± 0	90	216	± 45	115	313	+ 32 − 50
9	561	680	±147	399	568	± 34	581	889	±166
10	—	—	—	457	635	+112 − 86	519	827	+ 84 − 69
11	—	—	—	701	747	± 17	747	951	+129 −194
12	—	—	—	466	629	± 0	388	649	+138 − 94

A comparison of the figures calculated per 200 g. of body weight of the groups on CoP and −A diets shows that the direct influence of vitamin A deficiency was towards an increase in weight of these organs, on the average by 32 % (with a maximum of 57 % and a minimum of 12 %). This influence was absent only in litter 6. Since the influence of decreased food intake is opposed to that of vitamin A deficiency the influence on the weight of these organs would probably depend on the relative action of these two factors.

Adrenals.

No influence of decreased appetite or of vitamin A deficiency on the adrenals could be observed from the results obtained (Table IX) when the

Table IX. *Average weight of adrenals of rats in each group of each litter (mg.).*

	CoU diet			CoP diet			−A diet		
		Per 200 g. body wt.			Per 200 g. body wt.			Per 200 g. body wt.	
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation
1	42·0	29·0	±0·3	—	—	—	42·4	32·8	± 1·8
2	39·9	33·3	±3·8	—	—	—	31·2	34·4	+ 3·6 − 2·2
3	—	—	—	44·3	60·1	±2·0	39·7	65·1	+22·6 −11·6
4	—	—	—	48·3	46·1	±2·6	41·9	57·1	± 5·7
5	46·9	36·5	±0·8	48·1	40·6	±0·3	37·1	43·2	+ 8·6 − 7·8
6	—	—	—	31·6	51·4	±0·0	28·1	46·4	± 0·0
7	—	—	—	44·2	76·3	±6·2	34·8	70·7	+14·6 −10·6
8	33·6	70·7	±0·0	30·9	73·2	±1·6	26·3	75·8	+27·3 −19·3
9	42·5	51·5	±1·8	40·7	58·0	±3·9	29·3	45·4	± 0·2
10	—	—	—	42·9	60·1	+7·0 −6·9	31·6	50·4	+ 7·0 − 3·7
11	—	—	—	46·5	49·6	±1·0	38·2	45·9	+ 3·1 − 2·5
12	—	—	—	44·0	59·4	±0	29·4	49·0	+ 3·7 − 3·8

weights were calculated per 200 g. of body weight; the glands weighing, on the average, 57·5 mg. in the CoP group and 54·9 mg. in the −A group. The actual weights in all the litters except litter 1 were smaller in the −A group.

Thyroids.

A comparison of groups CoP and CoU in Table XI shows that decreased food intake produced in most of the rats of litters 5, 8 and 9 a more or less pronounced decrease in weight of the thyroids, both in the actual figures (on the average, 22 %) and when calculated per 200 g. of body weight (on the average 13 %). On −A diet the weights of the thyroids, in most cases in the actual figures, but always when calculated per 200 g. of body weight, were greater than those of the controls, on an average by 43 %.

This diet did not include iodine. Iodine was given to a pair of rats in each of the litters 10 and 12, in the daily amount of 7 γ, and to one pair of litter 11 in the daily amount of 30 γ. As can be seen from Table X, in these three litters the thyroids of the −A groups again weighed more than those of the controls when no supplementary iodine was given, but the same or even less when iodine was added to the diet.

Table X. *Average weight of thyroids of rats in each group of each litter (mg.).*

	CoU diet			CoP diet			−A diet			
		Per 200 g. body wt.			Per 200 g. body wt.			Per 200 g. body wt.		
Litter no.	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Actual wt.	Wt.	Deviation	Iodine content
1	28·4	19·7	±0·9	—	—	—	31·6	24·5	±1·3	−
2	25·3	20·9	+1·2 −1·0	—	—	—	25·0	27·5	+1·9 −1·6	− −
3	—	—	—	18·2	24·8	±2·4	21·6	35·1	+2·7 −1·5	− −
4	—	—	—	25·2	24·0	±0·6	24·8	33·7	±0·9	−
5	32·0	24·9	±1·5	24·5	20·8	±0·9	29·6	34·3	+4·7 −5·3	− −
6	—	—	—	13·0	21·1	±0·0	25·6	42·3	±0·0	−
7	—	—	—	15·4	26·2	±0·6	16·3	30·9	+5·0 −6·2	− −
8	16·0	33·6	±0·0	11·6	27·0	±1·0	16·0	43·9	+4·8 −6·1	− −
9	25·3	30·6	±5·0	20·9	29·7	±3·4	26·9	41·1	±7·7	−
10	—	—	—	19·9	27·7	+4·0 −2·2	23·0	36·0	±2·8	−
							17·5	28·6	±2·1	+
11	—	—	—	21·5	22·8	±2·3	22·7	28·5	±0·9	−
				—	—	—	19·0	24·4	±0·9	+
12	—	—	—	22·2	30·0	±0·0	20·1	32·6	±1·8	−
				—	—	—	14·4	23·4	±2·6	+

This indicates that the hypertrophy of the thyroids observed on our basal −A diet was produced, not by vitamin A deficiency, but by iodine deficiency. The control rats on CoU and CoP diets received sufficient iodine in the cod-liver oil which was given, this being one of the foods richest in iodine. Cotton-seed oil does not contain iodine in an amount detectable by micro-analysis.

Hypophysis.

In nearly all the litters, as can be seen from Table XI, the actual weight of the hypophysis was less in the −A and CoP rats than in those on complete unlimited diet. When, however, these weights are calculated per 200 g. of body weight, no significant or constant changes are observed, the weights being on the average 9·2 mg. and 8·7 mg. in the CoP and −A groups respectively.

Thymus.

No significant or constant changes were obtained in the weight of the thymus per 200 g. of body weight as a result of decrease in food intake in these experiments (Table XII).

In the rats on −A diet as compared with those on the CoP diet the weight of thymus per 200 g. of body weight in two (litters 8 and 12) of the 9 litters was not appreciably changed, whilst in the remaining seven it was less, on the average, by 23 % (maximum 38 %, minimum 12 %). The atrophic changes of the thymus in our experiments seem, therefore, to be related to the direct influence of vitamin A deficiency.

Table XI. *Average weight of pituitary of rats in each group of each litter (mg.).*

Litter no.	CoU diet			CoP diet			−A diet		
	Actual wt.	Per 200 g. body wt.		Actual wt.	Per 200 g. body wt.		Actual wt.	Per 200 g. body wt.	
		Wt.	Deviation		Wt.	Deviation		Wt.	Deviation
1	10·5	7·3	±0·5	—	—	—	9·6	7·5	±0·4
2	8·3	6·9	+0·4 −0·8	—	—	—	6·7	7·4	+0·2 −0·5
3	—	—	—	6·7	9·1	±0·4	5·8	9·4	+0·4 −0·7
4	—	—	—	7·2	6·9	±0·6	5·4	7·3	±0·4
5	9·1	7·0	±0·4	8·7	7·3	±0·7	6·3	7·2	+1·2 −1·3
6	—	—	—	5·2	8·4	±0·0	4·8	7·9	±0·0
7	—	—	—	6·6	13·0	±1·9	5·3	11·6	+7·2 −4·3
8	5·2	10·9	±0·0	5·0	12·0	±1·8	3·0	8·3	±0·1
9	6·8	8·3	±0·2	6·0	8·4	±0·2	7·1	10·9	±0·7
10	—	—	—	6·3	8·8	+0·2 −0·1	5·0	8·1	+1·2 −1·6
11	—	—	—	8·0	8·5	±0·1	6·1	7·7	+0·6 −1·2
12	—	—	—	6·8	9·2	±0·0	5·1	8·6	+1·4 −0·7

Table XII. *Average weight of thymus of rats in each group of litters nos. 4 to 12 (mg.).*

Litter no.	CoU diet			CoP diet			−A diet		
	Actual wt.	Per 200 g. body wt.		Actual wt.	Per 200 g. body wt.		Actual wt.	Per 200 g. body wt.	
		Wt.	Deviation		Wt.	Deviation		Wt.	Deviation
1	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—
4	—	—	—	317	302	± 8	182	249	± 76
5	580	453	±16	478	488	± 5	278	340	± 15
6	—	—	—	283	459	± 0	223	368	± 0
7	—	—	—	351	606	±51	200	374	+ 69 −105
8	226	475	± 0	160	378	±22	134	364	+ 54 − 49
9	377	457	±29	372	528	±13	235	360	± 44
10	—	—	—	159	225	+24 −41	125	198	+ 23 − 21
11	—	—	—	370	394	±26	268	341	+ 78 −123
12	—	—	—	227	307	± 0	184	309	+ 52 − 40

Iodine in −A diet.

We have already discussed the effect of addition of iodine to the −A diet on changes in the thyroid gland. No appreciable differences were noted, when iodine was included in the −A diet, in the changes above described in the other endocrine organs. Therefore, only in the case of the thyroid gland (Table X) in litters 10, 11 and 12 are the figures given separately for the groups fed on −A diet with and without the addition of iodine.

No influence was noted on the changes in body weight, gain per unit of food intake, or in fat deposition, or sexual organs.

Discussion.

It is clear from the data given above that, in rats kept on a vitamin A-deficient diet, two main factors are operating in influencing the organs and their functions.

The first is the decreased appetite, which, although directly caused by vitamin A deficiency, is in itself the cause of several secondary changes, which are entirely due to the effect of the decreased food intake.

The second factor is some other direct influence of vitamin A deficiency on the organs and their functions. In the animals kept on vitamin A-deficient diet the decreased food intake seemed to produce greater changes in fat deposition than in general body weight. For, while in our rats roughly 2/5 of the deficient gain in body weight was due to the decreased food intake, about 3/5 was caused by some other direct influence of the deficiency. In the fat deposition on the other hand these proportions were reversed, decreased food intake causing 3/5 of the deficiency in the body fat. This observation is in agreement with the general principle that with decreased food intake chiefly the carbohydrate and fat stores are affected first.

Measurements of body weight do not always represent the changes in growth of the animals, or the same degree of growth. In our young growing animals, however, these changes in weight roughly indicate the changes in growth. Thus in the following, with certain reservations, what is said about body weight also applies to the growth of the animals.

That the gain in weight is influenced to a considerable degree by the direct action of the deficiency is shown in all the litters in which vitamin A was depleted (*i.e.* litters 2 to 12). In every case one unit of the deficient diet produced a much smaller increase in body weight than the same unit of diet rich in vitamin A.

This property of vitamin A relating to building of tissue can be defined as an "anabolic principle." An increase in body weight and in growth of the animals is produced by this and by "the appetite producing" principle of this vitamin. In the absence of this "anabolic" principle, as is indicated by the results of our previous papers, the food consumed and absorbed is not deposited in the body normally or to a normal extent. The increased nitrogen

metabolism also testifies to this inability of the vitamin A-deficient organism to utilise the food normally for building new tissue.

In most cases the organs investigated were also under a twofold influence: that of the "appetite producing principle," *i.e.* of decreased food intake, and that of some other direct action of vitamin A deficiency.

With the sexual organs the unexpected result was noted of an increased weight, per 200 g. of body weight, of testes and notably of penis and prostate with seminal vesicles in most of the rats on −A diet as compared with those of the rats on CoP diet. In the case of the testes the oedematous condition would explain this increase, for histologically degenerative and atrophic changes were found in the seminiferous tissue which were completely absent from the testes of rats on CoP diet. In any case the changes in the testes did not affect the weights of the prostate with seminal vesicles or of the penis.

The decrease in food intake did not produce any significant changes in the weight (per 200 g. of body weight) of the penis, decreased the weight of the prostate with seminal vesicles and in some cases slightly increased the weight of the testes.

The thyroids in rats on −A diet were found to be hypertrophied. The −A diet, used by us, however, was deficient in iodine. When, in three litters, iodine was added to the −A diet no such hypertrophy was obtained. This change was therefore due to the iodine, rather than to the vitamin A, deficiency. In some rats decreased food intake on complete diet produced a more or less pronounced decrease in weight of the thyroids.

A direct influence of vitamin A deficiency on the thymus gland was evident as was shown by the decrease in weight of the gland.

No constant or significant changes were found in the adrenals and hypophysis.

Summary.

1. Using 63 male rats belonging to 12 litters a study was made of the influence of vitamin A deficiency on the body weight, fat deposition and the weight of the endocrine organs in order to distinguish the secondary changes produced by the decreased appetite from those produced by some direct influence of the deficiency.

2. Both these factors were found to be concerned in the changes produced in the organs and their functions.

3. At the stage of vitamin A depletion at which the rats were killed, about 2/5 of the deficiency in body weight would seem to be due to the decreased food intake, while 3/5 would seem to be caused by some other direct influence of the deficiency.

4. These proportions were reversed for the fat deposition.

5. In these experiments, in agreement with the previous results, it was found that one unit of the vitamin A-deficient diet produced a much smaller increase in body weight than was produced by the same unit of complete diet.

6. Therefore, vitamin A seems to have, in addition to an "appetite producing" principle, an "anabolic" principle, both of which are essential for building of the tissues, for increase in body weight and for growth of the animal.

7. When the weight of the organs is calculated per the same unit of body weight the following changes in the sexual organs were noticed. In most of the rats on vitamin A-deficient diet the weights of the testes and especially of the penis and of the prostate with seminal vesicles were greater than those of rats kept on a paired complete diet. The testes of the rats on the deficient diet seemed to be oedematous. The decreased food intake produced no noticeable effect on the penis, decreased the weight of the prostate with seminal vesicles and in some cases slightly increased the weight of the testes.

8. Reduction in the weight of the thymus was observed in most of the rats on the deficient diet and seemed to be due to the direct influence of vitamin A deficiency.

9. No significant changes were found in the weights of the adrenals or of the hypophysis.

10. The thyroids were hypertrophied only in the rats kept on the vitamin A-deficient diet which were used in the experiments. In rats receiving iodine in addition to this diet this change in the weight of the thyroids did not occur. It was, therefore, due to iodine deficiency.

11. No influence of iodine on any of the other changes caused by vitamin A deficiency was noticed.

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CLIX. THE INFLUENCE OF SUCCINIC, FUMARIC, MALIC AND ACETIC ACIDS ON THE DEPOSITION OF LIVER-GLYCOGEN.

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THE formation of succinic acid from pyruvic but not from acetic acid in muscle perfusion experiments led Toenniessen and Brinkmann [1930] to suggest this acid as an intermediate product in the oxidation of sugar *in vivo*. In the organism the part of succinic acid as an intermediate substance in the possible transformation of fat to sugar has been suggested by Clutterbuck and Raper [1925] and as a possible degradation product of the unsaturated acids by Smedley-MacLean and Pearce [1931]. Information as to the fate of succinic acid in the animal body is therefore of importance.

In the phloridzinised dog, feeding with succinic and malic acids resulted in the excretion of extra sugar [Ringer, Frankel and Jonas, 1913; Cremer, 1913] whereas feeding with malonic acid gave a negative result. Although Geelmuyden [1920] obtained extra sugar when acetic acid was administered to a phloridzinised dog, his results have not been confirmed by other workers [Deuel and Milhorat, 1928; Ringer and Lusk, 1910]. Thunberg's [1920] suggestion that succinic is formed from acetic acid in the body is therefore improbable. We investigated the effect of adding the ammonium salts of the four acids, succinic, fumaric, malic and acetic separately to a diet rich in fat and poor in carbohydrate which was fed to normal rats for 3 days following 24 hours' starvation. The glycogen and fat contents of the livers were then determined. In some experiments, a quantity of glucose of equal calorific value replaced the above acids.

The control diet and that to which sugar had been added were much better taken by the rats than the diets to which the salts of the acids had been added. It will be seen from Table I that in many cases in which the percentage of glycogen was increased the diet consumed was considerably less than in the controls. Rats about 6 weeks old were used, those for each experiment being from the same litter. The controls received a basal diet consisting of:

Bran (washed to remove most of the carbohydrate) ...	1·9 g.
Agar-agar	0·7
Bloater paste	0·5
Butter	3·0
Moisture	8·6
Total per diem ...	14·7

The experimental rats were given each day 2 g. of the ammonium salt of one of the acids to be tested in addition to the basal diet. The sugar controls received in addition to the basal diet 1·38 g. glucose, this being approximately equicalorific to 2 g. of ammonium succinate. After three days the rats were killed by a blow on the back of the head. The liver was rapidly removed, sliced, divided into two portions, one for estimating glycogen and one for fat: each portion was weighed and put into boiling 60 % KOH. The glycogen was precipitated, hydrolysed and estimated following the method of Evans, Tsai and Young [1931] the glucose being determined by the Shaffer-Hartmann method [1920–21]. The KOH solutions for fat estimations were acidified with hydrochloric acid, completely extracted with light petroleum and ether and the residues weighed. In order more accurately to determine the I.V. of the fats in Exp. 2, the acidified KOH solutions were extracted with carbon tetrachloride in the manner described by Hynd and Rotter [1930]. The extracts were made up to a definite volume and portions were titrated with $N/30$ alcoholic KOH, an equal volume of alcohol being added to the carbon tetrachloride solution before each titration. The fat content was calculated assuming a mean molecular weight for the fatty acids of 280.

The details of the experiments are shown in Table II and the collected results in Table I. The average glycogen percentages group themselves fairly sharply into three classes: (1) the percentages in the rats fed with the control diet and with the control diet *plus* acetate were respectively 1·08 and 1·26; (2) in those fed with the control diet to which succinate, malate or fumarate had been added, respectively 2·27, 2·18 and 2·18; (3) in those fed with the control diet and sugar the average percentage was 3·5. The *dl*-malate gave somewhat higher figures than the *l*-form. Clutterbuck [1927] had found that succinic acid incubated with minced liver yielded 75 % *l*-malic and 25 % fumaric acid. The fact that in our experiments *dl*-malic acid led to a higher percentage of glycogen in the liver than the *l*-form was therefore unexpected.

The average percentage of fat in the livers was markedly higher in the controls but no significant differences between the percentages in the other groups could be detected: the figures for the group which had received the additional sugar and for that which had received the acetate were similar.

Conclusion.

These results furnish evidence that in the normal rat the addition of succinic, malic or fumaric acid to a diet poor in carbohydrate leads to an increase of glycogen in the liver. After the addition of acetic acid no significant increase in glycogen could be detected.

The feeding experiments were carried out by Miss E. M. Hume and Miss Henderson Smith, whose co-operation and help we gratefully acknowledge.

We desire to acknowledge a grant from the Department of Scientific and Industrial Research which made it possible to carry out this work.

Table I.

No. of exp.	Diet (basal) (wt. in g. per day)			% glycogen in the liver						% fat in the liver					
	Carbo-hydrate	Protein	Fat (butter)	Control	Acetate	Fuma-rate	Malate	Suc-cinate	Sugar control	Control	Acetate	Fuma-rate	Malate	Suc-cinate	Sugar control
1	0·910	0·327	3	1·31 ♀	0·89 ♀	—	—	—	—	—	—	—	—	—	—
				1·30 ♀	1·54 ♀	—	—	—	—	—	—	—	—	—	—
					1·74 ♀	—	—	—	—	—	—	—	—	—	—
2	—	—	—	1·18 ♀	2·55 ♂	—	—	2·46 ♂	—	*5·27 ♀	4·47 ♂	—	—	4·07 ♂	—
				0·95 ♂	—	—	—	—	—	7·43 ♂	—	—	—	—	—
				0·22 ♀	—	—	—	—	—	11·05 ♀	—	—	—	—	—
3	—	—	—	—	0·74 ♀	—	*l-* 1·72 ♀	1·49 ♀	2·11 ♀	—	6·89 ♀	—	*l-* 5·16 ♀	4·95 ♀	4·15 ♀
					1·09 ♀	—	*l-* 2·31 ♂	1·45 ♂	0·65 ♀	—	5·89 ♀	—	*l-* 3·50 ♂	3·31 ♂	4·68 ♀
4	0·937	0·326	3	—	2·00 ♂	—	*l-* 1·05 ♀	0·10 ♀	1·63 ♀	—	4·81 ♂	—	*l-* 5·69 ♀	7·98 ♀	9·29 ♀
					0·56 ♀	—	*l-* 2·31 ♂	2·63 ♂	2·28 ♀	—	6·92 ♀	—	*l-* 4·16 ♂	5·17 ♂	7·11 ♀
5	0·950	0·347	3	1·88 ♀	—	0·58 ♀	*dl-* 3·42 ♂	4·67 ♂	4·88 ♀	10·16 ♀	—	4·60 ♀	*dl-* 3·65 ♂	3·07 ♂	6·69 ♀
						2·13 ♂	*l-* 1·41 ♀	—	4·20 ♀	—	—	3·49 ♂	*l-* 4·51 ♀	—	4·34 ♂
6	0·948	0·350	3	1·06 ♀	—	2·89 ♂	*dl-* 2·16 ♂	1·35 ♀	2·98 ♀	7·76 ♀	—	3·12 ♂	*dl-* 4·35 ♂	4·64 ♀	5·42 ♀
						2·23 ♀	*l-* 2·38 ♂	—	5·04 ♀	—	—	5·88 ♀	*l-* 3·79 ♂	—	5·32 ♀
7†	0·805	0·339	3	0·76 ♀	0·22 ♀	3·05 ♀	*dl-* 3·44 ♂	2·38 ♀	3·44 ♀	7·24 ♀	5·27 ♀	4·43 ♀	*dl-* 4·81 ♂	1·87 ♀	3·80 ♀
						2·20 ♂	*l-* 1·63 ♀	4·06 ♀	4·31 ♀	—	—	2·70 ♂	*l-* 4·99 ♀	4·61 ♀	5·10 ♀
Mean	0·910	0·338	3	1·08	1·26	2·18	2·18	2·27	3·50	8·15	5·71	4·04	4·46	4·41	5·59

* By CCl_4 extraction and titration.

† Albinos; all the other litters were black and white.

Table II.

(Protocols.)

No. of exp.	Diet	Sex	Original weight g.	% loss of weight	% food eaten	% glycogen	% fat
1	Control	♀	90	3	100	1·31	
	Control	♀	78	2	100	1·30	—
	Acetate	♀	78	6	84	0·89	—
	Acetate	♀	94	7	72	1·54	—
	Acetate	♀	95	6	66	1·74	—
2	Control	♀	120	4	100	1·18	5·27*
	Control	♂	139	6	100	0·95	7·43
	Control	♂	105	3	100	0·22	11·05
	Acetate	♂	157	6	100	2·55	4·47
	Succinate	♂	149	9	94	2·46	4·07
3	Acetate	♀	102	9	98	0·74	6·89
	Acetate	♀	103	4	92	1·09	5·89
	Succinate	♀	105	10	82	1·49	4·95
	Succinate	♂	104	16	88	1·45	3·31
	l-Malate	♂	120	5	99	1·72	5·16
	l-Malate	♂	110	13	82	2·31	3·50
	Sugar control	♀	97	6	63	2·11	4·15
					(rat pneumonia)		
	Sugar control	♀	98	5	98	0·65	4·68
4	Acetate	♂	118	8	86	2·00	4·81
	Acetate	♀	119	7	90	0·56	6·92
	Succinate	♀	130	13	60	0·10	7·98
	Succinate	♂	130	12	44	2·63	5·17
	l-Malate	♂	125	7	90	1·05	5·69
	l-Malate	♀	138	9	78	2·31	4·16
	Sugar control	♀	120	4	100	1·63	9·29
	Sugar control	♀	121	0	100	2·28	7·11
5	Control	♀	70	2	98	1·88	10·16
	Succinate	♂	83	9	87	4·67	3·07
	dl-Malate	♂	80	9	66	3·42	3·65
	l-Malate	♀	75	9	78	1·41	4·51
	Fumarate	♀	82	12	57	0·58	4·60
	Fumarate	♂	76	9	69	2·13	3·49
	Sugar control	♂	72	1	99	4·88	6·69
	Sugar control	♂	70	0	91	4·20	4·34
6	Control	♀	105	7	100	1·06	7·76
	Succinate	♀	120	9	68	1·35	4·64
	dl-Malate	♂	140	12	49	2·16	4·35
	l-Malate	♂	138	11	79	2·38	3·79
	Fumarate	♀	115	10	49	2·89	3·12
	Fumarate	♀	117	10	79	2·23	5·88
	Sugar control	♀	115	2	100	2·98	5·42
	Sugar control	♀	110	0	100	5·04	5·32
7†	Control	♀	108	2	100	0·76	7·24
	Acetate	♀	112	4	84	0·22	5·27
	Succinate	♀	101	4	94	2·38	1·87
	Succinate	♂	108	5	72	4·06	4·61
	dl-Malate	♂	125	6	84	3·44	4·81
	l-Malate	♀	123	5	92	1·63	4·99
	Fumarate	♀	113	4	80	3·05	4·43
	Fumarate	♂	120	6	68	2·20	2·70
	Sugar control	♀	108	Gain of 2	100	3·44	3·80
	Sugar control	♀	103	Gain of 2	92	4·31	5·10

* In this group fats were estimated by extraction with carbon tetrachloride and titration with sodium hydroxide.

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CLX. THE PHOSPHATIDES OF FORAGE GRASSES. I. COCKSFOOT.

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A recent investigation by Channon and Chibnall [1927] of the phosphatides of cabbage leaves has shown that lecithin and kephalin are absent, and that the main constituent is a new phosphatide, calcium phosphatidate. In the preliminary investigations of the grasses the methods of these workers for the preparation and purification of leaf phosphatides were applied, but were not very successful for two reasons. In the first place the grass blades contained a higher proportion of solids, and especially of fibre, than cabbage leaves, consequently the grinding operations necessary to prepare the ether extract from the fresh material were very laborious; further, the percentage of phosphatide in the ether extract was low, so that 20 kg. samples of fresh grass gave only 2–4 g. of crude phosphatide. In the second place the treatment necessary for the preparation of pure phosphatidic acid resulted in the formation of emulsions. These are to be expected when working with any extract containing phosphatides, but in the present case they were far more difficult to break than those which had been encountered during the purification of the cabbage phosphatides. The presence of phosphatidic acid was readily proved, but the yields were disappointingly low, and attempts to separate and purify it by taking advantage of the water-solubility of the sodium salt showed that a large proportion of the crude phosphatide fraction was non-acidic in character, and was therefore essentially different from that found in the cabbage.

This result was to a certain extent unexpected, because in these early stages of the research we were working with products prepared from perennial ryegrass (*Lolium perenne*) and the analysis of the crude material was similar to that obtained from cabbage. (Found: C, 54·5; H, 8·3; ash, 19·8; Ca, 5·4; P, 3·9 %. A typical cabbage preparation gave C, 57·2; H, 8·7; ash, 19·5; Ca, 5·9; P, 4·6 %.) Later batches of material, however, from this and other grasses, often gave lower figures for ash, calcium and phosphorus, and in addition significant amounts of magnesium and nitrogen, *e.g.* batch *R* (Cocksfoot) gave C, 52·8; H, 8·1; ash, 12·5; P, 2·4; Ca, 1·0; Mg, 1·4; N, 1·0 %. These results, coupled with the observation that obstinate emulsions were

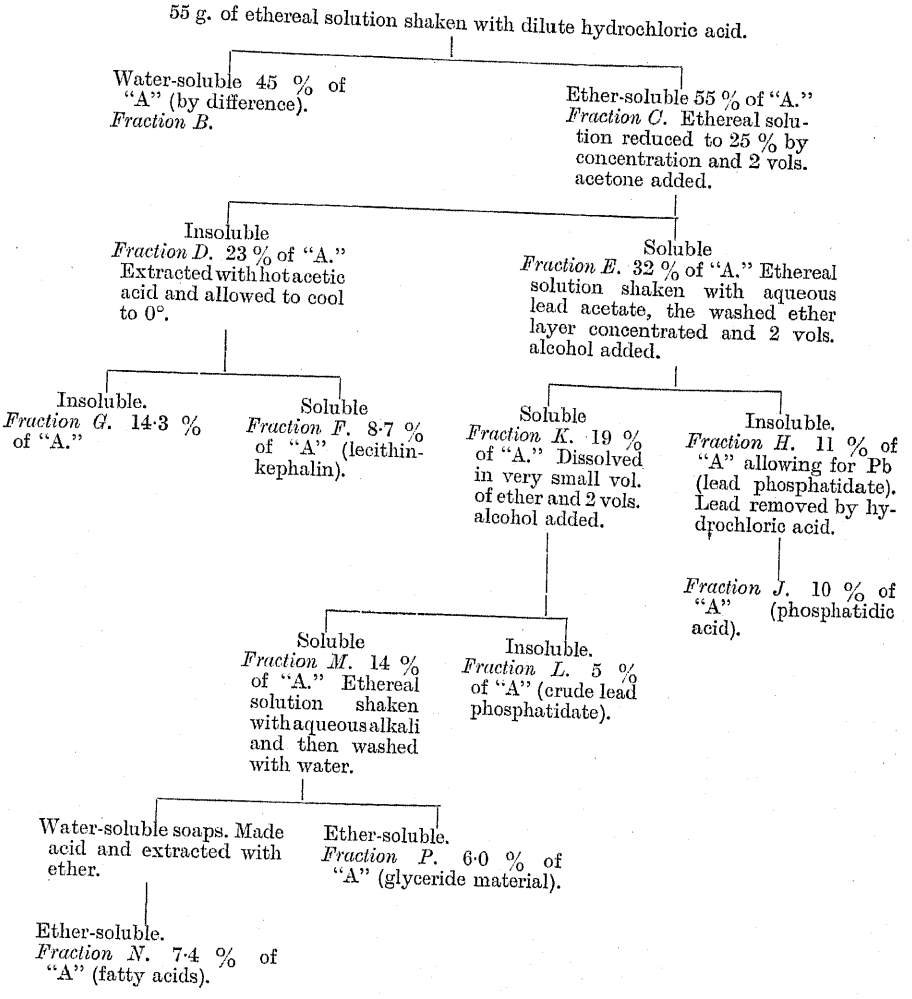
always formed when an ethereal solution of crude phosphatide was shaken with hydrochloric acid, led us to suspect the presence of lecithin and kephalin. To separate these two phosphatides from phosphatidic acid successfully demanded a larger quantity of crude phosphatide than we had hitherto been able to prepare by the methods we were then employing, and a change of procedure was necessary.

Now in connection with the general research which is being made in this laboratory into the composition of the ether extract of grasses we had already made numerous experiments to find out the best method of preparation; whether, for instance, the direct extraction of dried grass was preferable to the procedure of Chibnall and Channon [1927]. These results will be reported more fully in another communication, but at the above-mentioned stage in the phosphatide research it had been found that good yields of crude phosphatide could be obtained from grasses that had been dried very rapidly (1–2 hours) in a steam-oven through which a slow current of air was drawn, whereas the yields from grass dried slowly at room temperature or at 40° were poor. But the rapid drying of, say, 150 kg. of grass under these conditions was beyond the equipment of the laboratory, and we could not make use of dried grass as our raw material until we were fortunate enough to have placed at our disposal the new experimental grass-drying plant of the Imperial Chemical Industries at Jealott's Hill, in which the grass, supported on a moving belt, is passed along a chamber through which air heated initially to about 200° is blown. Drying is effected in about 8 minutes without "scorching" of the grass or any apparent chlorophyll breakdown. We were then able to obtain 22 kg. of dried cocksfoot (*Dactylis glomerata*), and from this 56 g. of crude phosphatide (Batch *R*). Knowing the extreme lability of phosphatides, as evidenced by the history of the "cuorin" and "jecorin" of the earlier workers in this field, we were apprehensive as to the fate of the grass phosphatides under this admittedly drastic drying treatment, and the procedure was adopted more or less under duress because it seemed at the time to be the only one available.

The large batch of crude phosphatide was readily fractionated by the method outlined in Table I, and we had no difficulty in showing that fraction F consisted chiefly of lecithin and kephalin by isolating derivatives of choline and β-aminoethyl alcohol after acid hydrolysis. The elementary analysis of this fraction showed that whereas the ratio N : P was practically 1 : 1 the actual amounts of N and P present were lower than were to be expected if it consisted only of these two phosphatides. This was found to be due, at any rate in part, to the presence of sugars or substances giving sugars on acid hydrolysis, which, calculated as glucose, were equivalent to 6·1 % of the fraction. This apparent adsorption of sugar by the phosphatide material will be referred to again later.

Having removed the major part of the lecithin and kephalin, and also fraction G, which will be shown later to consist of decomposition products, it

Table I. *Fractionation of crude phosphatide (fraction A).*



was possible to isolate phosphatidic acid by precipitation as the lead salt in the usual way (fractions H and J). We were thus able to obtain definite proof that the three phosphatides lecithin, kephalin and phosphatidic acid (as Ca or Mg salt) were all present in the blades of cocksfoot, which must, therefore, differ materially in their phosphatide metabolism from cabbage leaves, which contained only calcium phosphatidate.

While this result was satisfactory we were disappointed to find that the lecithin and kephalin (fraction F) and phosphatidic acid (fractions H and L) accounted for only about 9 % and 15 % respectively of the crude phosphatide (fraction A). An amount equal to 45 % passed into the aqueous phase on shaking with acid and the remainder into the three fractions G, N and P

which we believe to be products of decomposition brought about by the heating necessary to dry the grass and the subsequent treatment with aqueous acid. This conclusion is based partly on the exhaustive researches of Thudichum, MacLean and Levene, who, given adequate quantities of material, have been able to show that crude animal phosphatides contain only lecithin, kephalin and sphingomyelin, and that numerous other so-called individual phosphatides consist of lecithin and kephalin mixed with decomposition products which have been produced by the treatment necessary to prepare and purify the phosphatide material. Were it not for the fact that calcium phosphatidate was found by Channon and Chibnall to be the only phosphatide present in cabbage leaves, and that we have evidence, to be published shortly, that the lecithin and kephalin present in runner bean seeds give place first to magnesium and later to calcium phosphatidate during germination, we should have hestitated to suggest that fractions H and L represented phosphatidic acid and were not themselves products of kephalin decomposition.

Fraction G was a light grey powder insoluble in all organic solvents other than chloroform, and except that it had a N : P ratio nearly 1 : 1 instead of 1 : 2 resembled in other respects the "cuorin" isolated by Levene and Rolf [1926] from soya bean seed crude phosphatide. After acid hydrolysis it gave 8·1 % of reducing material calculated as glucose and only 20 % of fatty acids soluble in light petroleum. All the nitrogen was in the amino-form, and the fraction appeared to consist of kephalin fragments mixed with other products which had been rendered ether-soluble by the presence of true phosphatides in the original ether extract. Fraction P consisted of glycerides and fraction N of fatty acids. Both these fractions were freely soluble in ether-acetone, and could not have existed as such in the original ether extract; otherwise it is difficult to understand how they could have been precipitated by acetone with the waxes and phosphatides, and in any case have escaped extraction with the waxes during the hot acetone treatment. It seems reasonable to assume that they had been split off by the hydrochloric acid from phosphatide fragments which still retained the acetone-insolubility of the true phosphatides themselves. A study of the component fatty acids (as shown in Table II) lends support to this contention, for the acids from fractions N and P resemble those of the phosphatides F and J more closely than they do those derived from the glycerides of the true fat.

The mixed fatty acids from each of the four fractions F, J, N and P were oxidised under controlled conditions. Tetrahydroxystearic (sativic) acid and hexahydroxystearic (linusic) acid were readily isolated, showing that the original acids contained linoleic and linolenic acids. In a previous paper [Smith and Chibnall, 1932] we discussed at some length our inability to obtain any crystalline dihydroxystearic acid from the oxidation products of the true glyceride fatty acids of cocksfoot and perennial ryegrass and came to the tentative conclusion that if an octadecadienoic acid were present in the glycerides it was not oleic acid. Our experience in the oxidation of the phos-

Table II. *Oxidation of the fatty acids from various fractions of the cocksfoot phosphatides.*

Fraction	F (Lecithin-kephalin) %	J (Phospha-tidic acid) %	N (Free fatty acids) %	P (Glyceride material) %	Fatty acids from glycerides of true fat [Smith and Chibnall, 1932] %	Fatty acids from linseed oil %
Saturated acids	19	33	28	34	14	14
Dihydroxystearic acid fraction	3·3 (gum)	2·8 (gum)	5·4 (gum)	4·6 (gum)	3·6 (gum)	12·7 (crystalline M.P. 131°)
Tetrahydroxystearic acid	12·1	17·2	10·0	7·0	4·1	5·4
Hexahydroxystearic acid	4·3	6·7	9·0	10·0	3·4	5·9
Wt. of acids taken for oxidation (g.)	1·16	1·8	2·59	1·48	11·0	2·05
I.V. of acids taken for oxidation	144	128	137	125	185	176

phatide fatty acids again leads to the same conclusion. In each case the dihydroxystearic acid fraction consisted of a small amount of gum from which no crystalline material other than minute amounts of tetrahydroxystearic acid could be isolated. To obtain definite evidence that our technique was not at fault we oxidised a similar quantity of fatty acids derived from linseed oil, and isolated dihydroxystearic acid, M.P. 131°, without any difficulty. We therefore conclude that oleic acid is not present in the cocksfoot phosphatide fatty acids. It is interesting to note that Levene and Rolf [1925; 1926] in their analyses of the constituent fatty acids of soya bean lecithin and "cuorin" were unable to identify oleic acid.

The low proportion of saturated acids in the mixed fatty acids from cocksfoot phosphatides is in keeping with the results of Levene and Rolf for soya bean and of Channon and Chibnall for cabbage leaves, and emphasises the fact that plant lecithin and kephalin differ from those derived from animal sources in that they do not contain saturated and unsaturated acids in equimolar proportions.

The persistence with which most of these phosphatide fractions retain reducing sugars recalls the similar observations of Schulze and his co-workers, who worked with crude phosphatide preparations from seeds. Levene and Rolf [1925; 1926] were able to show, however, that in the case of the soya bean phosphatides this sugar was an impurity which could be removed by drastic purification, showing that no cerebroside or sugar-containing phosphatide was present. Sufficient evidence has been collected to suggest that this is also the case with the grass phosphatides.

When the crude phosphatide (fraction A) was shaken in ethereal solution with hydrochloric acid, material equivalent to 45 %, including reducing sugars equivalent to 8·1 %, passed into the aqueous phase. Fraction G on acid

hydrolysis gave 11 % and fraction F 6 % of reducing sugars, equivalent to 1·6 % and 0·5 % of fraction A respectively. Fraction A therefore contained at least 10 % of reducing sugars. The values for the sugar content of other batches of cocksfoot examined during the course of this work are collected in Table III. It will be observed that in the two cases in which the crude

Table III. *Sugar content of certain cocksfoot phosphatide fractions.*

Batch number	Treatment of grass	Solvent used for extraction	Reducing sugars in crude phosphatide (calculated as glucose) %
Q 1930	Fresh method of Chibnall and Channon [1927]	Ether	0·9
	Rapidly dried	Ether	3·1
A 1931	Fresh method of Chibnall and Channon [1927]	Ether	0·7
	Rapidly dried	Ether	3·0
	Rapidly dried	Light petroleum B.P. 40–60°	3·7

phosphatide was prepared by the procedure of Channon and Chibnall the sugar content was low, and when it is remembered that this method removes most of the water-soluble products, and therefore most of the leaf sugars, before the "coagulum" and residual leaf material are extracted with ether to prepare the crude phosphatide, it seems legitimate to assume that in the case of the dried grasses the sugar has been taken into ethereal solution through the agency of the lecithin and kephalin, which are known to possess unique solubility properties.

To summarise the results of our investigations on the crude phosphatide of rapidly dried grass, we have been able to show that it contains lecithin, kephalin and a salt of phosphatidic acid. Considerable amounts of decomposition products, probably derived from kephalin are also present, and for this reason it does not seem to us profitable to discuss either the relationship which the amount of crude phosphatide isolated bears to the amount of true phosphatide originally present in the fresh grass, or whether the phosphatidic acid was present as calcium or magnesium salt.

Experimental.

Before describing the fractionation of the phosphatides prepared from cocksfoot it is necessary to discuss briefly the theoretical analytical figures which are to be assumed for the lecithin, kephalin and phosphatidic acid present.

Investigation of the constituent fatty acids from each of the products isolated has shown that the I.V. was 125–145 and that approximately one-third was composed of saturated fatty acids M.P. 53–55°, the analytical figures for which in every case fall between those of palmitic acid $C_{16}H_{32}O_2$ (C, 74·9;

H, 12·6 %; mol. wt. 256), and stearic acid $C_{18}H_{36}O_2$ (C, 76·0; H, 12·8 %; mol. wt. 284). The saturated acids from the glycerides of the true fat [Smith and Chibnall, 1932] were similar and were shown to consist of about 70 % palmitic, 20 % stearic and 10 % higher acids. If therefore 25 % of the total acids from the three phosphatides be taken as palmitic acid, then it is probable that the remaining 75 % consists of C_{18} acids whose mean unsaturation is equivalent to three double bonds in two molecules, and it is on this basis that the following analytical figures have been calculated.

	C %	H %	N %	P %
Lecithin	65·3	10·9	1·77	3·91
Kephalin	65·6	10·6	1·91	4·24
Phosphatidic acid	66·5	10·4	—	4·52
Lead phosphatidate	51·2	7·7	—	3·48

Lead phosphatidate having this composition should give an ash of lead pyrophosphate equal to 33 %.

During the present research more than one hundred micro-combustions have been carried out on products obtained at various stages in the fractionation, and we have found it unusually difficult to obtain reproducible figures for carbon, hydrogen and ash. In almost all cases the carbon figure was liable to vary between 1 and 2 units in 50 or 60, while the ash showed a similar but not necessarily corresponding difference.

The following are quoted as typical examples:

		C %	H %	Ash %
Crude lead phosphatidate (Batch *P*)	1	50·3	7·8	30·5
	2	48·2	7·1	29·5
The same, after purification by precipitation from ethereal solution by alcohol	1	47·9	7·6	32·9
	2	47·0	7·6	32·6
	3	48·3	7·4	32·6
Theory requires		51·2	7·7	33·0

Throughout the investigation reported below we have not been able to handle sufficient material to enable us to prepare chemically pure lecithin, kephalin or phosphatidic acid. We have, therefore, been dealing throughout with mixtures, and the errors may be due, in large part, to the difficulty of obtaining a representative sample of such gummy substances in the small amount required for a micro-combustion. Loss of ash may also be caused by the creeping of the molten material from the boat before it is completely burnt.

Fractionation of the crude phosphatide from dried cocksfoot.

22 kg. of the dried cocksfoot (*Dactylis glomerata*) were exhaustively extracted with ether by British Drug Houses, Ltd. and gave 770 g. of ether extract. This was dissolved in 2 litres of warm ether and 4 litres of hot acetone were added with stirring. After standing overnight at 0° the precipitate of

wax and phosphatide was filtered off, redissolved in a similar quantity of warm ether and again precipitated with acetone. The resulting mixture was then extracted six times with hot acetone to dissolve out the wax fraction, each acetone extract being filtered hot. As the crude phosphatide was a sticky gum which might well retain some of the wax, it was thought advisable, after the second and fourth extractions with hot acetone, to dissolve the residue in ether and remove the solvent by evaporation, thereby exposing a new surface of the phosphatide to the action of the hot acetone. No appreciable amount of phosphatide passed into the hot acetone, although, as will be shown later, it contained both lecithin and kephalin. The crude phosphatide thus obtained (fraction A, Table I) weighed 56 g., equivalent to 7·3 % of the ether extract. It analysed as follows: C, 52·8; H, 8·1; P, 2·44; Ca, 1·02; Mg, 1·36; N, 1·0; ash, 12·5 %. The subsequent treatment of the material is outlined in Table I.

Treatment of the crude phosphatide with dilute hydrochloric acid.

55 g. of "A" were dissolved in ether, shaken four times successively with 500 cc. of dilute hydrochloric acid, and then well washed with water. Emulsification at this stage caused considerable trouble, but by varying the relative amounts of ether and of water present, by the judicious addition of small amounts of alcohol, and by the aid of the centrifuge, this difficulty was largely overcome. 55 % of the original material remained soluble in ether, so that by difference 45 % had passed into the acid and aqueous washings. These latter were collected and analysed. They contained 79 % of the calcium, 76 % of the magnesium, 41·5 % of the phosphorus and 26 % of the nitrogen originally present in the crude phosphatide. In addition, reducing substances, estimated as glucose by the method of MacLean [1919] were present to the extent of 8·1 % of the crude phosphatide.

The ethereal solution on evaporation gave 30 g. of material which analysed as follows: C, 63·0; H, 9·3; P, 2·73; Ca, 0·28; Mg, 0·12; N, 0·63; ash 3·9 %. This was dissolved in the minimum amount of ether and several volumes of acetone added. After standing overnight at 0° the precipitate was centrifuged and washed with acetone. The ether-acetone solution and the acetone washings were collected, the solvents removed, the resulting material again dissolved in the minimum amount of ether and precipitated with acetone. The material insoluble in ether-acetone constitutes the impure lecithin-kephalin fraction D, and the soluble material the crude phosphatidic acid mixture E.

Fraction D. This fraction represented 23 % of fraction A, and analysed as follows: C, 53·4; H, 8·8; ash, 9·3; P, 2·82; N, 1·5 %. It was extracted three times with boiling acetic acid, the extracts united, reduced to a small volume and allowed to stand at 0° for some hours. The material soluble in the cold acetic acid represents the lecithin-kephalin fraction F, and the material insoluble in both hot and cold acetic acid constitutes fraction G, which will be shown later to consist of decomposition products.

Lecithin-kephalin (*fraction F*). This fraction, representing 8·7 % of the original fraction A, was a black sticky gum which analysed as follows: C, 63·5; H, 10·1; P, 3·0; N, 1·4; ash, 6·0 %; N : P ratio 1·03 : 1. It was shown to consist chiefly of lecithin and kephalin by identification of the products obtained on acid hydrolysis.

The material (2·3 g.) was hydrolysed by boiling with 5 % sulphuric acid for 8 hours. The cooled mixture was then exhaustively extracted with ether, which removed 1·59 g. of fatty acids. The aqueous solution and washings were reduced *in vacuo* to 100 cc. and analysed for nitrogen and reducing sugars. The total N was 25 mg., the amino-N was 8 mg. and the non-amino-N (by difference) was 17 mg. Reducing substances, estimated as glucose by the method of MacLean [1919] were 140 mg. or 6·1 %. Glucose, and especially fructose, when submitted to the hydrolysis mentioned above would be partially destroyed, so that the low values for phosphorus and nitrogen in this lecithin-kephalin preparation are due, at any rate in large part, to the presence of carbohydrate.

Identification of β-aminoethyl alcohol. A sample of synthetic β-aminoethyl alcohol, kindly provided by Dr Dudley, was found to be quantitatively precipitated by the mercury carbamate method employed by Neuberg and Kerb [1912] for amino-acids, and the aqueous hydrolysis products of fraction F were accordingly treated in the following way.

Sulphuric acid was removed from solution with baryta, the barium sulphate being centrifuged and well washed with hot water. Lead acetate was then added, the small precipitate centrifuged, and lead removed from the clear liquid by means of hydrogen sulphide. The water-clear solution thus obtained was concentrated to 100 cc. and treated alternately with small amounts of 20 % aqueous sodium carbonate and 20 % aqueous mercuric acetate. The reaction was kept just alkaline to litmus and the reagents added until the addition of either produced a permanent orange precipitate. An equal volume of alcohol was then added and the mixture allowed to stand overnight. The precipitate was centrifuged off, washed with a little 50 % alcohol, suspended in water and mercury removed with hydrogen sulphide. The mercuric sulphide was well washed with hot water, the clear liquid and washings acidified with a little hydrochloric acid and evaporated to dryness *in vacuo*. The hydrochloride of β-aminoethyl alcohol was then extracted from the residue of inorganic salts with alcohol. The alcoholic extract was run into a small beaker, evaporated to dryness on the water-bath, the residue taken up in a few cc. of dilute hydrochloric acid and gold chloride added. The mixture was allowed to evaporate slowly in a vacuum desiccator. Brown cubic crystals came out during the course of a few days, and long orange needles were obtained on recrystallisation from water. The melting-point alone, and when mixed with authentic β-aminoethyl alcohol chloroaurate was 189–191° with previous softening at 187°. (Found: Au, 48·7. $C_2H_7ON.HAuCl_4$ requires Au, 49·2 %.)

Identification of choline. The aqueous-alcoholic filtrate from the mercuric

acetate precipitation mentioned above was concentrated *in vacuo* to 20 cc. and treated with a hot concentrated alcoholic solution of mercuric chloride. A white crystalline precipitate appeared almost at once, and increased on cooling. The mother-liquor on concentration gave another crop of crystals, and the total yield was 1·3 g. The crystals were typical of choline mercuric chloride and melted at 252° with previous softening. For confirmation 0·5 g. of the material was dissolved in a few cc. of hot water, mercury removed with hydrogen sulphide and the calculated amount of gold chloride added to the clarified liquid. Fine yellow needles of choline chloroaurate appeared on standing, and were filtered off. The mother-liquor on concentration gave a further yield of beautiful yellow plates. Both needles and plates melted at 269–270° with decomposition. (Found: C, 13·9; H, 3·2; Au, 44·6. $C_5H_{14}ON.AuCl_4$ requires C, 13·5; H, 3·2; Au, 44·5 %.) Allowing for aliquots withdrawn for analysis the yield of choline mercuric chloride represented 70 % of the non-amino-N present in the hydrolysis products of fraction F.

Identification of the fatty acids. The 1·59 g. of fatty acids mentioned above (I.V. 144; mol. wt. 290) represented a yield of 69 %. The theoretical yields for lecithin and kephalin are 69 % and 75 % respectively. For further identification of the constituent acids of this and other mixed fatty acids derived from the crude phosphatide the acids were oxidised with dilute alkaline permanganate below 10° as described by Lapworth and Mottram [1925]. The oxidation was allowed to proceed for 5 minutes when it was stopped by decolorising with sodium bisulphite followed by a little concentrated hydrochloric acid. The oxidation products insoluble in water were filtered off, dried in a vacuum desiccator, and the saturated acids extracted with light petroleum (B.P. 80–100°). These were then subjected to the magnesium soap treatment of Bertram [1927], to give an approximate value for the amount of saturated acid present in the original mixed fatty acids. The residue insoluble in light petroleum was next extracted three times with boiling ether to remove any dihydroxystearic acid. The material thus obtained in this and other similar fractions from the crude phosphatide was a brown gum, and attempts to prepare crystalline material resulted in the isolation of minute amounts of tetrahydroxystearic acid, whereas in the case of linseed oil, crystalline dihydroxystearic acid, M.P. 131°, was obtained without difficulty. The material insoluble in ether was recrystallised from alcohol and in each case was found to consist of mixed α- and β-sativic acids. The oxidation products which remained soluble in water were obtained by evaporating the solution to dryness *in vacuo* and extraction with alcohol. In all cases either α- or β- or both linusic acids were obtained.

In the case of the mixed fatty acids from fraction F, 1·16 g. were oxidised and gave (1) 0·21 g. of saturated acids which was recrystallised from alcohol. (Found: C, 74·5; H, 12·6 %; mol. wt. 282; M.P. 53–55°.) (2) 0·04 g. of dark gum representing the dihydroxy-derivatives. (3) 0·09 g. of tetrahydroxystearic acid M.P. 155–165°. (Found: C, 62·2; H, 10·7. $C_{18}H_{36}O_6$ requires C,

62·0; H, 10·4 %.) Fractionation from ethyl acetate gave α- and β-sativic acids of M.P. 157–159° and 172–173° respectively. (4) 0·05 g. of hexahydroxystearic acid. M.P. 185–190°. By repeated recrystallisation from water α-linusic acid, M.P. 203–204°, was obtained. (Found: C, 56·9; H, 9·7. $C_{18}H_{36}O_8$ requires C, 56·8; H, 9·5 %.)

Phosphatidic acid (fraction J).

The crude phosphatidic acid (fraction E) mentioned above represented 32 % of the crude phosphatide (fraction A). The analysis, C, 68·5; H, 10·3; P, 2·02; ash, 2·6 %, showed that some other fatty material, possibly glyceride, must have been present. This mixture, dissolved in ether, was shaken four times with a strong solution of lead acetate. The ethereal solution was well washed with water, concentrated to a small bulk and three volumes of alcohol added. The precipitate of lead phosphatidate was centrifuged, redissolved in a similar volume of ether and again precipitated with alcohol. The weight of lead salt (fraction H) so obtained, allowing for the lead present, represented 11 % of the crude phosphatide (fraction A). (Found: C, 50·7; H, 7·5; ash, 31·4. Lead phosphatidate requires C, 51·2; H, 7·7; ash, 33·0 %.) An ethereal solution of the salt was shaken with dilute hydrochloric acid to remove the lead, washed with water, and evaporated to dryness *in vacuo*. The amount of phosphatidic acid (fraction J) was equivalent to 10 % of the crude phosphatide (fraction A). (Found: C, 63·9; H, 9·8; ash, 6·6; P, 4·24; N, 0·6. Phosphatidic acid requires C, 66·5; H, 10·4; P, 4·52, N, 0 %; mol. wt. 686.) The I.V. was 98 and the mol. wt. by titration 746, showing that the preparation still contained some non-acidic material. This was confirmed when 3·46 g. were saponified with strong alcoholic potassium hydroxide for 2½ hours. 1·91 g. of mixed fatty acid, representing only 77 % of the theoretical yield, were obtained. The I.V. was 128 and the mol. wt. 298.

Identification of the fatty acids. 1·80 g. of mixed fatty acids were oxidised by the methods outlined above, and gave (1) 0·60 g. of saturated acids, M.P. 52·5–54·5°. (Found: C, 75·6; H, 13·0 %.) (2) a gum weighing 0·06 g. representing the dihydroxystearic acid fraction. (3) 0·31 g. of mixed α- and β-sativic acids, which were separated by means of ethyl acetate. (4) 0·12 g. of hexahydroxystearic acid. Several recrystallisations from water and alcohol gave products melting at 197° and 167° respectively. (Found, for the latter: C, 56·8; H, 9·8 %.) The melting-points of α- and β-linusic acids are stated to be 203° and 173° respectively and in view of the small amount of material available it is probable that the fractionation had not been carried far enough.

Phosphatide decomposition products.

Under this heading are described the three fractions which appear to be products of phosphatide decomposition brought about by heat during the drying of the cocksfoot.

Fraction G. This fraction was insoluble in glacial acetic acid, and represented 14 % of the original crude phosphatide (fraction A). It was a light grey powder which absorbed chloroform to form a gel but which did not readily disperse into solution. Otherwise it was insoluble in the usual organic solvents. (Found: C, 51·0; H, 8·3; ash, 3·5; P, 2·15; N, 1·1 %.) The product resembled in many respects the so-called "cuorin" fraction which Levene and Rolf [1926] isolated from soya bean seed phosphatides, which was soluble only in chloroform, had a ratio N : P of 1 : 2 and which they considered to be a mixture of kephalin decomposition products. In the present case the decomposition had been more extensive and the product was contaminated with substances giving reducing sugars on acid hydrolysis.

4·4 g. of material were hydrolysed by boiling with 10 % hydrochloric acid for 8 hours, the mixture cooled and extracted with ether. 0·25 g. of a black powder remained insoluble in both ether and water, while the ether extracted 1·31 g. of material which was molten at 100° and semi-solid at room temperature. This latter gave 0·92 g. of fatty acids soluble in light petroleum, a yield of only 21 %, showing that the phosphatide fragments had lost part of their complement of fatty acids. The aqueous acid contained reducing substances equivalent to 0·48 g. of glucose and also 0·043 g. of nitrogen, all of which was in the amino-form.

Fraction N (free fatty acids). Fraction K, the mother-liquor from the precipitation of the lead phosphatidate (fraction H), was concentrated to a small volume and three volumes of alcohol added. A further quantity of crude lead phosphatidate (fraction L) was precipitated. (Found: C, 55·1; H, 8·3; ash, 23·5 %.) The clear alcoholic solution (fraction M) represented 14 % of the crude phosphatide (fraction A). (Found: C, 73·8; H, 11·1; ash, 1·2 %.) The analysis suggested that the product was a mixture of fatty acids or glycerides. 5·20 g. were accordingly dissolved in ether and shaken with aqueous sodium hydroxide. 2·25 g. of neutral material (fraction P) remained in the ether and 2·76 g. of fatty acids (fraction N) were recovered from the alkaline soap solution. 2·59 g. of these acids were oxidised and gave (1) 0·72 g. of saturated acids (Found: C, 75·4; H, 12·4 %; mol. wt., 284; M.P. 53·5–55·5°); (2) 0·14 g. of dark gum representing the dihydroxystearic acid fraction; (3) 0·21 g. of tetrahydroxystearic acid, M.P. 165° indefinite (Found: C, 61·8; H, 10·5. $C_{18}H_{36}O_6$ requires C, 62·0; H, 10·4 %); fractionation from ethyl acetate and alcohol gave α- and β-sativic acids, of M.P. 158–160° and 173° respectively; (4) 0·27 g. of crude hexahydroxystearic acids; M.P. 184° with previous softening at 165°.

Fraction P (mixed glycerides). The neutral material from the above separation represented 6 % of fraction A. (Found: C, 74·9; H, 11·8 %.) On mild saponification 2·1 g. gave 1·63 g. of mixed fatty acids (I.V. 125; mol. wt. 280) and 0·25 g. of unsaponifiable material, probably a wax alcohol. (Found: C, 81·3; H, 12·8 %.) The saponification value was 167, or, allowing for the small amount of unsaponifiable material, 185. These results suggest that the

saponifiable material consisted chiefly of diglyceride fragments split off from the phosphatide. A monoglyceride requires C, 70·7; H, 11·3 %; sap. val. 156: a diglyceride C, 75·4; H, 11·7 %; sap. val. 180: and a triglyceride C, 77·1; H, 12·3 %; sap. val. 189.

Oxidation of 1·49 g. of the fatty acids gave (1) 0·51 g. of saturated acids (Found: C, 75·1; H, 12·8 %; M.P. 59–60°; mol. wt. 259); (2) 0·07 g. of gum representing the dihydroxystearic acid fraction; (3) 0·105 g. of mixed α- and β-sativic acids, M.P. 157–163°, (4) 0·145 g. of mixed α- and β-linusic acids, M.P. 173°. The composition of these glyceride fatty acids, and also of the free fatty acids of fraction N, more closely resembles that of the phosphatides than that of the glyceride components of the cocksfoot fat (compare Table II).

Oxidation of fatty acids from linseed oil. 2·35 g. of linseed oil gave on saponification 2·17 g. of mixed fatty acids of I.V. 176. 2·05 g. of these acids were oxidised and gave (1) 0·29 g. of saturated acids, M.P. 52–53°, (2) 0·26 g. of dihydroxystearic acid; recrystallised repeatedly from alcohol it melted at 130·5–131° (Found: C, 68·3; H, 11·4; $C_{18}H_{36}O_4$ requires C, 68·5; H, 11·5 %); (3) 0·11 g. of mixed α- and β-sativic acids, M.P. 168–170°; (4) 0·12 g. of mixed α- and β-linusic acids, M.P. 192°.

SUMMARY.

The phosphatide fraction prepared from a sample of rapidly dried cocksfoot has been investigated in some detail.

Considerable phosphatide decomposition appears to have occurred during the drying, but lecithin, kephalin and the Mg or Ca salt of phosphatidic acid have been definitely identified.

The constituent fatty acids of the phosphatides have been investigated by controlled oxidation. Saturated acids, linoleic acid and linolenic acid were present. As in the case of the glyceride fatty acids of cocksfoot, no evidence for the presence of oleic acid could be obtained.

The ratio of saturated to unsaturated acids was less than unity, as appears to be usual in the case of plant phosphatides.

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CLXI. THE GONADOTROPIC HORMONES (ρ-FACTORS).

III. FURTHER PURIFICATION AND PROPERTIES OF THE ACTIVE PRINCIPLES.

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Since the original observation by Zondek [1930] that the gonadotropic substances of pregnancy urine are precipitable by alcohol in sufficient concentration, numerous processes, based on this and other observations, have been devised by other workers with the object of obtaining a highly active substance in such a condition that it could be stored without loss of potency. Dickens [1930] made preparations by the alcohol precipitation process, and also by methods based on precipitation with ammonium sulphate or tannic acid. He obtained a product which, on many occasions, had a luteinising action in the immature mouse in doses of 0·015 mg., and in one instance only of 0·006 mg. Wiesner and Marshall [1931] by considerable modification of the alcohol precipitation process also obtained a product of comparable activity on many occasions, and in one instance by a process based on the precipitation of the active factors with phosphotungstic acid.

Fischer and Ertel [1931], in a comprehensive communication, describe various attempts at purification of commercial samples of the gonadotropic hormones which contained 4–5 m.u. per mg.; they obtained products containing, in some cases, as much as 130 m.u. per mg. by removal of phosphates with baryta or barium acetate, a method which was previously used by Wiesner and Marshall [1931], who, however, removed the barium more completely by the use of sulphuric acid. Some measure of purification was also obtained by Fischer and Ertel by adsorption of the active principles on kaolin or bolus alba at p_H 4·5 and elution with dilute ammonia. Electrodialysis at temperatures $< 10°$ of a product containing 120 m.u. per mg. failed to effect further purification and even caused a destruction of active substances.

Marshall [1931] has shown that solutions of ρ-factor preparations can be selectively filtered through a series of "membran" and "ultrafein" filters supplied by the Membranfilter Gesellschaft, the coarser grades passing the active factors completely and the finer only to a small extent, if at all. In

the course of this work it was observed that even in those cases where the ρ-factors were almost quantitatively retained by the filter, the filtrate was quite strongly coloured. This observation suggested the possibility of removing a large part of the coloured organic impurities in this way, such impurities being, incidentally, the cause of the original hygroscopic nature of the products. The filters which seemed likely to be of use in this connection were "ultrafein mittel," "ultrafein fein," and "ultrafein Benzopurpurindicht." The former two were actually used in the experiments to be described.

The possibility of further purification *via* the phosphotungstates was also investigated but the results obtained were far inferior to those from the filtration technique.

Attempts at purification by combination with permutit in acid solution, with subsequent extraction with dilute ammonia were entirely unsuccessful.

Animal standardisation.

In order to determine the potency of different preparations, various dilutions were made in 0·85 % saline and injected in convenient amounts into several series of 5 mice each. The total volume injected into each animal was 0·25–0·50 cc. and this was divided into 5 doses given over a period of $2\frac{1}{2}$ days. With the highly active products the solution injected contained 0·01 mg. of solid substance per cc. After 2–3 days following the last injection the animals were killed, a positive result being regarded as one in which at least two animals of a series showed an ovarian response (*i.e.* corpora lutea or atretica). In some cases, as will be seen later, cornification of the vaginal epithelium was observed with a dose less than that required to produce an ovarian effect.

It should be borne in mind that accurate standardisation is out of the question where so many different factors are involved. This is particularly the case when one considers that, on present conceptions, there are two factors responsible for the effects upon the ovary, and that these factors are antagonistic to an extent which we are as yet unable to measure quantitatively. At the present time, although separation of ρ_1 and ρ_2 cannot be effected, we have no certainty that the relative proportions of the two are constant in different preparations. Although some such approximate standardisation is necessary in the absence of a more satisfactory method, its limitations must be recognised and an endeavour made to standardise at least the factors which can be controlled, such as the number of injections into which a dose is divided and which have been shown to exercise such a great effect in the "standardisation" of oestrin (α).

EXPERIMENTAL.

Experiments with permutit. A solution of 1·0 g. ρ-factors (20 units per mg.) in 10 cc. water was acidified to p_H 4·5 with sulphosalicylic acid, shaken vigorously at intervals with permutit and allowed to stand for half an hour. The permutit was removed by centrifugation, washed with water acidified with

sulphosalicylic acid, and extracted with 10 cc. of 1 % ammonia (sp. gr. 0·880, 1 : 100) by shaking. After centrifugation the supernatant fluid was neutralised with 10 % acetic acid and 4 volumes of absolute alcohol and 1 volume of ether were added. The precipitated product was washed with alcohol, then with ether, and dried. A dose of 0·025 mg. failed to produce any ovarian response in the immature mouse. No attempt was made to determine whether the product was active in larger doses, since the sole object of the experiment was to investigate the possibility of utilising the method as a basis for further purification.

Experiments with phosphotungstic acid. A solution of 1·0 g. ρ-factors (20 units per mg.) in 9 cc. of water and 1 cc. of 10 % sulphuric acid was centrifuged to remove a small amount of insoluble matter, and fractional precipitation resorted to. Fraction *a* was produced by addition of 2 cc. of 20 % phosphotungstic acid; fraction *b* by addition of 3 cc. of phosphotungstic acid to the supernatant fluid from *a*; fraction *c* by further addition of 5 cc. of the acid to the supernatant fluid from *b*. All three fractions were washed with absolute alcohol and dried. They were then suspended in water; solution was effected by cautious addition of 10 % ammonia (sp. gr. 0·880; 1 : 10), and phosphotungstic acid removed with barium hydroxide. The excess of barium was quantitatively removed with sulphuric acid and the active ρ-factors were precipitated by addition of 4–5 volumes of alcohol. Washing and dehydration were effected by means of alcohol and ether as previously described. Fractions *a* and *b*, tested in immature mice, were found to contain 40 units per mg. Fraction *c* was almost inactive.

Filtration experiments. The products used in the filtration experiments were all entirely free from protein and usually had a potency of about 20 units per mg.; they were prepared by the alcohol precipitation method, involving the use of barium hydroxide, previously described by Wiesner and Marshall [1931] for the preparation of their *E* 198. The following is a résumé of the technique which has been employed in some dozen instances. A solution of 2 g. of ρ-factors in 100 cc. of distilled water (adjusted to p_H 7·2) was filtered at the water-pump on two filters ("ultrafein mittel" or "fein"), the effective filtering area of which was about 7 cm. in diameter. When the volume of residual liquid on each filter was reduced to 5–10 cc., distilled water was added and the filtration allowed to proceed until the residual volume was not more than 5 cc. This washing process was repeated three times, by which time the filtrate was colourless. The residual liquid was thoroughly washed off the surface of the filters and the solution centrifuged until quite clear. Addition of 5 volumes of absolute alcohol gave merely an opalescent solution, but the purified ρ-factors were precipitated by the further addition of an equal volume of ether. The operation up to this point took about 7–8 hours. The precipitate was removed by centrifugation, washed with alcohol and ether, and dried. The yield was usually 0·15–0·2 g. The potency of the product, in every case in which filtration was as complete as described above, was 200 units

(luteinising) per mg. It is interesting to note that in two experiments 60 % of the animals receiving 0·0025 mg. of active substance showed complete cornification of the vaginal epithelium, the vaginae being well open in all cases 3 days after the cessation of injections. This means that the oestrogenic potency of these samples is 400 units per mg. and it also confirms Wiesner and Marshall's findings with regard to the differential effects of small and large doses with cruder preparations, although in this series of experiments, which deals with liminal doses as regards luteinising activity, the vaginae were open without exhibiting cornification in the larger doses. In the course of this work it has been noticed that the filtrates frequently possessed a certain degree of activity. This may be due to two causes, (1) the relatively concentrated solutions used for filtration (400 units per cc.) whereby activity in the filtrate was more readily observed in the doses given, and (2) the slightly increased permeability of the membranes after continued use. A product was obtained from these filtrates which was compared for its nitrogen content with the highly active substance remaining on the filter.

Chemical investigation of the products obtained.

Nitrogen content. Determinations of nitrogen were made on a considerable number of specimens using a micro-Kjeldahl method. Merck's perhydrol (0·5 cc.) was used to assist the oxidation as it was found to be very effective for this purpose, whilst containing only minute traces of nitrogen. Table I gives the values obtained with the original product, the product from the filtrate and the highly active residue on the filter.

Table I.

Substance	N content %	Activity (units per mg.)
Original product (102)	5·2 5·3	*ca.* 20
Filtrate (102 *b*)	4·6 4·6	>2
Filtrate (102 *e*)	4·4 4·4	<2
Active residue (102 *f*)	8·4	200
Active residue (102 *a*)	8·3 8·5	200
Active residue (102 *d*)	7·9 8·1	145

The figures given are consistent with the hypothesis that the active substance is a N-containing compound, the highest percentage being found in the most active product, and the lowest in the relatively inactive product from the filtrate. Specimen 102 *d* was submitted to less efficient washing in the process of filtration and has therefore a somewhat lower activity than 102 *a* and *f*. Many other specimens with an activity of 200 units per mg. gave similar results. No allowance has been made in the above figures for ash content of the specimens. No. 102 *f* had an ash content of 7·16 %, the residue

containing no calcium or barium and only a minute trace of iron; the N content on an ash-free basis is therefore 9·09 %. Fischer and Ertel [1931] give N values of 10·0–11·10 % on an ash-free basis for a product with an activity of 120 units per mg.

Qualitative tests (on the highly active product).

(1) No halogens, phosphorus, or sulphur are present.

(2) No pentoses (phloroglucinol and Bial's tests) are present.

(3) Millon's test is definitely positive.

(4) Molisch's test gives a colour which is much redder than that given by glucose.

(5) Phosphotungstic acid, in presence of sulphuric acid, gives a flocculent white precipitate which is biologically definitely active.

(6) The glyoxylic acid reaction is very doubtful.

(7) Addition of 5 volumes of alcohol alone to a relatively concentrated solution (2000 m.u. per cc.) does not precipitate the active factors. Further addition of ether or a small volume of saturated sodium chloride solution is required; in actual practice ether was always used.

(8) The ninhydrin reaction for α-amino-acids is negative alone, but following brief boiling with dilute sulphuric acid and subsequent neutralisation the reaction is obtained.

(9) Knoop's bromine test for histidine is negative.

Tyrosine and tryptophan content. An approximate tyrosine and tryptophan determination was made on the highly active product by Folin and Marenzi's method [1929]; results: tyrosine, 1·25 %; tryptophan, 0·6 %. Owing to the small amount of material which remained after the completion of other experiments, it was not possible to repeat these analyses with extraction of the alkaline hydrolysates with toluene, which procedure was found by Macfarlane and Fulmer [1930] to be necessary for obtaining accurate tryptophan values with certain proteins. The figure recorded above is, therefore, almost certainly too high, and the rather doubtful qualitative glyoxylic acid reaction obtained is explained.

Attempt to demonstrate the presence of carbohydrate. A partially purified specimen (20 units per mg.) was first used and the following technique adopted [*cf.* Osborne and Campbell, 1900]. 1 g. of the product (20,000 m.u.) was boiled under reflux with 20 cc. of 10 % sulphuric acid. After hydrolysis the sulphuric acid was removed quantitatively with barium hydroxide and the precipitate of barium sulphate and humin centrifuged off. The precipitate was washed once with 10 cc. of distilled water. The clear supernatant fluid (p_H 7·0–7·5) was evaporated to 10 cc. and 1·0 g. of sodium acetate and 0·5 g. of phenylhydrazine hydrochloride were added. The hot solution was immediately filtered from some tarry material and then further heated at 100° for 1 hour and allowed to cool overnight. The precipitate was collected and washed with water and a little 95 % alcohol. To the solution in boiling alcohol

1–2 parts of hot water were added and the solution was allowed to cool slowly. The yellow-brown product which separated had the typical crystalline form of glucosazone. After a further crystallisation the product had M.P. 183–185°; yield 10 mg.

A similar technique using 0·4 g. (80,000 m.u.) of the highly purified product failed to give any trace of osazone. Fischer and Ertel [1931] were also unable to obtain an osazone from a product with an activity of 100–120 m.u. per mg.

Attempt at crystallisation. 2 cc. of saturated ammonium sulphate were added to 0·1 g. of highly active ρ-factors in 1 cc. of water (p_H 7·0). After standing for 2 days at 0° some amorphous product had separated but no crystalline material could be detected.

Biological experiments.

In addition to the standardisation of preparations already referred to, a number of preparations were tested for their efficacy in inducing ovulation in the rabbit. With samples of which 1 m.u. = 0·005 mg. it was found that 0·1 mg. would produce rupture of one or more follicles with probable ovulation although ova were not isolated. It is proposed later to determine whether any relationship exists between the mouse unit (luteinising) and the rabbit unit (ovulation) with a view to obtaining evidence of the identity, or otherwise, of the factors. This point is of particular interest in view of Leonard's [1931] suggested differentiation.

Discussion.

The filtration technique described above has been found extremely useful in the preparation of small quantities of a highly active, almost salt-free product. The use of the ultrafiltration method also enabled the author to remove some coloured resinous impurities which could not be removed by ordinary dialysis through collodion or cellophane membranes. As a result of this work it has been made clear that the precipitability of ρ-factors from protein-free solutions by alcohol is dependent on at least a small inorganic salt content, since sodium chloride can replace ether as a precipitating agent for the opalescent solutions obtained by the addition of alcohol alone. The fact that the ninhydrin reaction was only obtained after a brief hydrolysis with mineral acid is important as support for the view that the product is polypeptide in nature. When, in previous work, a qualitatively very definite positive Millon's reaction was obtained, it seemed possible that tyrosine formed an intrinsic part of the active molecule, as was also suggested by Fischer and Ertel. The roughly quantitative determinations reported here would seem to cast some doubt on this hypothesis, although the presence of a polypeptide has been more or less definitely established. Further, that such polypeptide, if it may be assumed to be the active principle (or principles), does not possess its activity merely by virtue of the presence of the fundamental —CO—NH— linkages which it contains, is obvious from the finding that a solution of

purified ρ-factors loses its entire activity on heating at 100° for half an hour, whereas such heating does not decompose the polypeptide sufficiently to give the ninhydrin reaction. If, therefore, our assumption that the active principles are polypeptide in nature is correct, we must of necessity postulate some other rearrangement of the molecule or splitting off of some side-chain in a key position without effect on the polypeptide linkage. The only other alternative would be that the active principles of unknown nature were adsorbed on the polypeptide.

It seems hardly likely that any polysaccharide forms an essential part of the molecule, as suggested by Fischer and Ertel, in view of their own failure to obtain any osazone following either brief or prolonged hydrolysis, but also by reason of the author's demonstration of the presence of a sugar in a partially purified sample (containing 20,000 m.u.) coupled with his inability to do so in a highly purified sample containing 80,000 m.u.

Summary.

1. A further purification of ρ-factor preparations by an ultrafiltration method is described. "Ultrafein mittel" and "fein" filters of the Membranfilter Gesellschaft are used. Specimens containing 200 m.u. per mg. have been obtained.

2. Neither the use of phosphotungstic acid nor that of permutit yielded sufficiently satisfactory results.

3. The presence of a polypeptide in the highly purified product is established by the application of the ninhydrin reaction before and after hydrolysis.

4. The presence of a sugar complex in the active substance is doubtful.

5. Not more than 1·25 % of tyrosine is present in the purified product which also contains about 8·4 % of nitrogen and 7 % of ash. The presence of tryptophan is very doubtful.

6. 0·1 mg. of the product will induce rupture of the follicles with probable ovulation in the rabbit within 24 hours.

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CLXII. HISTAMINASE.

I. THE HISTAMINE-HISTAMINASE REACTION. II. PURIFICATION. III. SPECIES DISTRIBUTION.

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Histaminase is the name which has been applied [Best and McHenry, 1930] to a histamine-inactivating enzyme. Originally found by Best [1929] to be present in ox and horse lung, investigations on the histaminase content of dog tissues showed it to be most plentifully contained in the kidney and in the intestinal mucosa. These studies, utilising fresh tissue *in vitro*, led to perfusion experiments in which it was found that isolated dog kidneys could inactivate large amounts of histamine. The obvious suggestion has been made that this enzymic mechanism, functioning principally in the kidney and in the wall of the small intestine, may constitute one of the body's protections against harmful amounts of histamine.

Some of the properties of histaminase have been previously described. It is heat-labile, being irreversibly inactivated at 60°. For its activity the optimal temperature is 38°, and the optimal p_H range is from 6·9 to 8·0. The enzyme is inhibited slightly by ammonium and magnesium ions, and markedly by calcium, while it is not affected by sodium and potassium. Of the anions, chloride is without effect, sulphate and citrate are inhibitory, and phosphate definitely augments the reaction. As it was found that the histamine-histaminase reaction does not occur in presence of $M/2000$ potassium cyanide, proceeds very slowly in an atmosphere of nitrogen, and is accelerated by oxygen, it has been thought to be oxidative. Under certain conditions, if not always, the enzyme causes disruption of the iminazole ring in the histamine molecule.

Employing the Barcroft-Warburg technique, Gebauer-Fuelnegg and Alt [1932] have measured oxygen consumption during the histamine-histaminase reaction. The enzyme was prepared from dog kidney. No simple molar relation was demonstrated between the amount of oxygen consumed and the amount of histamine inactivated. Three possible discrepancies were noted by these authors between their conclusions, based on oxygen-uptake measurements during the histamine-histaminase reaction, and results obtained from other methods of investigation, which had been reported from this laboratory:

(1) in their experiments more histamine was inactivated at p_H 10 than at 7·4; (2) more histamine was inactivated at 50° than at 37°; (3) potassium cyanide did not completely inhibit the reaction. Since the publication of these results we have repeated the measurements of inactivation at varying temperatures and at varying hydrogen ion concentrations and have found no disagreement with our earlier results.

Several observations by Gebauer-Fuelnegg and Alt are of interest. The enzyme seems to be specific for histamine, as indicated by the lack of oxygen-uptake when active kidney-powder was incubated with several other iminazole derivatives. We had previously found that histaminase does not affect tyramine, an amine possessing the same side-chain as is found in histamine. The results of the brief study by Gebauer-Fuelnegg and Alt of species distribution of histaminase are not in agreement in the case of several species with the data presented in the present paper.

This communication gives results of further studies on the histamine-histaminase reaction, descriptions of some purification procedures and data on the variation in the histaminase contents of kidneys of several species of animals.

As a source of histaminase, a dry powder prepared from ox kidney by extraction of the fats with acetone, removal of acetone with ether and subsequent drying of the tissue was employed in earlier experiments. One powder prepared in this manner showed no loss of activity during storage for one year at laboratory temperature. To secure a stable powder it is essential that the acetone extraction be thorough and that the powder be rapidly dried to remove ether. More recent work has shown that hog kidney is a better source and histaminase powder is now prepared from that tissue by the method described above. The enzyme is removed from the powder by extraction at 38° with phosphate buffer of p_H 7·0 to 7·2. The amount of enzymic action is determined by measuring the amount of histamine remaining after incubation of a mixture containing a known amount of histamine and the tissue or enzyme preparation. All determinations of histamine have been made by the blood-pressure method in etherised cats. For the assays solutions were prepared from the mixtures after incubation by the hydrochloric acid procedure [Best and McHenry, 1930].

I. The histamine-histaminase reaction.

The results of preliminary work on the rate of the reaction indicated that (1) after mixture of certain proportions of histamine and enzyme, there was, previous to actual inactivation, a phase during which no change in histamine, as shown by the depressor activity, occurred; (2) when the quantity of histamine used was constant, the total amount of amine destroyed depended on the concentration of enzyme; (3) the amount of histamine inactivated by a constant amount of enzyme was not markedly affected by the histamine

concentration. The second and third observations have been confirmed by Gebauer-Fuelnegg and Alt.

More accurate measurements of the rate of inactivation have been made in an attempt to elucidate the nature of the changes occurring. The general method was as follows: a solution of histaminase prepared by extracting kidney powder with phosphate buffer was added to a solution of histamine acid phosphate in phosphate buffer at p_H 7·2. Toluene was used as a preservative and the mixture was kept, with continuous agitation by means of a stirrer or a current of gas, at 38° in a water-bath. Samples for histamine determinations were removed at intervals through a siphon dipping beneath the toluene.

In the first series the proportion of enzyme to histamine was such that the amine would be inactivated in about 24 hours. A histaminase solution was prepared by extracting 30 g. of ox kidney powder with 270 cc. phosphate buffer, p_H 7·2, for one hour with continuous agitation. After filtration 75 cc. were added to phosphate buffer, p_H 7·2, containing 30 mg. histamine acid phosphate (10 mg. base) the volume was made up to 500 cc. with buffer and 50 cc. of toluene were added. There was a definite preliminary period of 5 hours during which no change in the depressor activity of the solution could be detected. The values secured during the second, inactivating phase lie fairly well on a smooth curve (Fig. 1). On semilogarithmically ruled paper the inactivation phase is represented by a straight line. The values of k in the equation $k = \frac{1}{t} \log \frac{a}{a-x}$, where a = initial amount of histamine and x = amount inactivated, have been calculated. When this is done, using for t values from the initial admixture, k is not a constant. If the values of t be corrected by subtracting the length of the lag phase from the elapsed time, k is constant (Table I). As judged from these values for the velocity constant, the reaction is unimolecular.

Table I. *Velocity constant of histamine-histaminase reaction.*

t	$t_1 = t - 5$	a	$a - x$	$k = \frac{1}{t_1} \log \frac{a}{a-x}$
6 hrs.	1	10 mg.	8·5 mg.	0·071
8 ,,	3	10 ,,	6·4 ,,	0·065
10 ,,	5	10 ,,	4·7 ,,	0·066
12 ,,	7	10 ,,	3·5 ,,	0·065
14 ,,	9	10 ,,	2·5 ,,	0·067
16 ,,	11	10 ,,	1·6 ,,	0·072

Determinations of a similar nature have been carried out with varying proportions of enzyme. These all showed the two phases. The duration of the lag phase was inversely proportional to the concentration of histaminase, and the rate of the inactivation phase was directly proportional to the concentration. In each case the rate of the second phase was logarithmic.

Earlier work had shown that in the absence of oxygen and when nitrogen or hydrogen was present almost exclusively the histamine-histaminase reaction

did not occur. Determinations of the rate, similar to those previously made, were carried out with large amounts of oxygen bubbling through the mixture. In these the length of the lag phase was one-half that found without the stream of oxygen but with the same concentration of enzyme and of histamine. No great change was observed in the second phase. In another experiment three sets of measurements were made on the same day using portions from one solution of enzyme in each set. Through one mixture air was bubbled, through the second oxygen and through the third nitrogen. The results are shown in

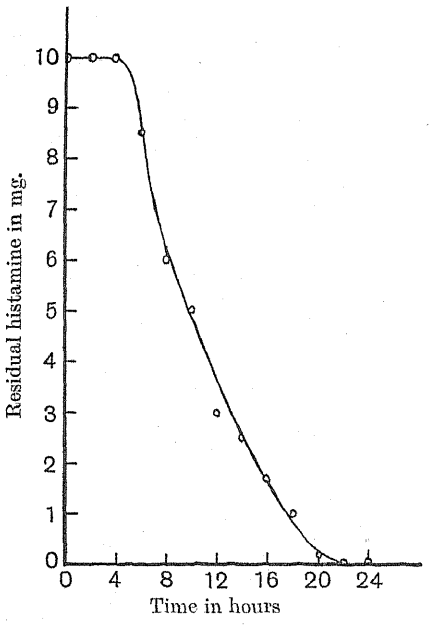


Fig. 1. Rate of inactivation of histamine.

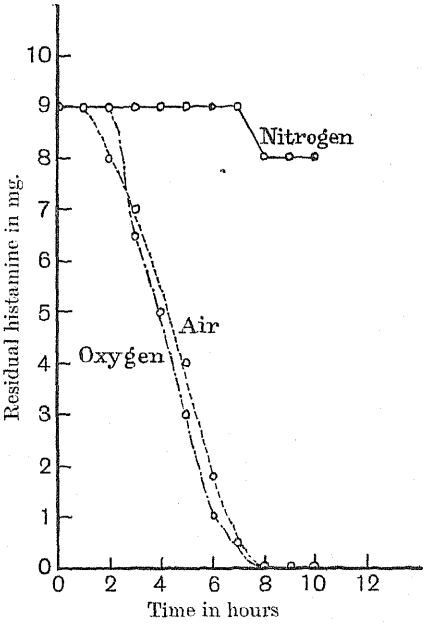


Fig. 2. Effect of nitrogen and oxygen upon histaminase.

Fig. 2. The rates for air and oxygen are practically identical but nitrogen definitely slowed the reaction, scarcely any inactivation occurring.

It had previously been found that low concentrations of potassium cyanide prevented the inactivation of histamine. It seemed advisable to determine whether the addition of cyanide to a previously cyanide-free mixture in which inactivation was progressing would stop any further action of the enzyme. For this three flasks were used. Each flask contained 75 cc. histaminase solution (the filtrate secured after extraction for one hour of 60 g. of ox kidney powder with 540 cc. phosphate buffer), 30 mg. histamine acid phosphate (10 mg. base), sufficient buffer of p_H 7·2 to make a volume of 500 cc. and 50 cc. toluene. The results are shown in Fig. 3. In the first case 0·002 *M* potassium cyanide was initially present and no inactivation occurred. In the second case an equal amount of cyanide was added when 25 % destruction had taken

place, and it is evident that further inactivation was inhibited. The third curve demonstrates that this particular enzyme preparation was acting in the customary manner. In view of the results of Gebauer-Fuelnegg and Alt [1932] which suggested incomplete inhibition by cyanide it is interesting that we have repeatedly found cyanide to prevent the initiation of the inactivation and to cause its cessation when added at an intermediate stage.

Since cyanide had an inhibitory effect upon the enzyme it seemed desirable to investigate the action of sodium pyrophosphate. In a concentration of 0·05 *M* at p_H 7·2 this salt has no effect upon the histamine-histaminase reaction. As has been suggested by Gebauer-Fuelnegg and Alt, iron is probably excluded as an active agent, while copper may be a factor.

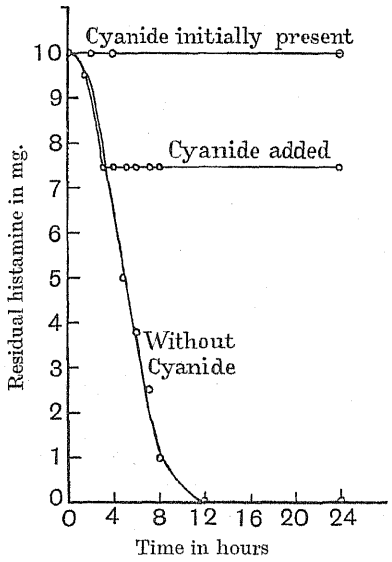


Fig. 3. Effect of cyanide upon histaminase.

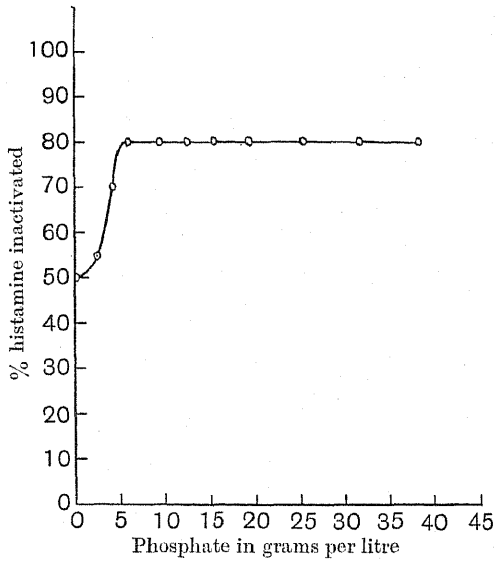


Fig. 4. Effect of concentration of phosphate upon histaminase.

Of the various ions investigated, phosphate alone has a definitely accelerating effect upon the action of histaminase. This has been studied quantitatively and the results are given in Fig. 4. Phosphate is not essential for the reaction, which proceeds in the absence of this ion, but the amount of histamine inactivated by a constant amount of enzyme in a constant time is increased when phosphate is present. There is no definite concentration of phosphate which may be regarded as optimal, rather there is an optimal range. The amount of phosphate present in our routine measurements of histaminase action lies within this range.

The changes produced in histamine by histaminase are of considerable interest. In the case of the amine, tyramine, Ewins and Laidlaw [1910] found that, after removal of the amino-group from the side-chain, the remnant was

oxidised to the alcohol and then to the acid. Guggenheim and Loeffler [1916] showed that similar reactions occurred in the cases of phenylethylamine, indolethylamine, and *iso*amylethylamine. They were unable to isolate iminazolyl acetic acid after inactivation of histamine. In view of the destruction of the iminazole ring previously reported [Best and McHenry, 1930], the failure of Guggenheim and Loeffler in the case of histamine is explicable. In experiments with tyramine and tyramine oxidase conducted *in vitro*, Hare [1928] found that deamination occurred but that only half the nitrogen present in the tyramine molecule could be accounted for as ammonia. In an extension of this work [Bernheim, 1931] the same author showed that the course of oxidation of tyramine varied under different conditions. Under certain conditions, *p*-hydroxyphenylacetic acid was produced, and in this case there was complete deamination of tyramine. A quantitative study of ammonia production was not completed. To gain some information on the changes produced in histamine, measurements have been made of ammonia produced during the reaction.

To determine the amount of ammonia various methods have been tried. The procedure found most useful was, after the customary incubation of a mixture of kidney powder and histamine in phosphate buffer, to filter this mixture through Whatman No. 41 paper. The ammonia content of a 10 cc. aliquot portion of the filtrate was measured, using the Van Slyke and Cullen aeration apparatus. In nearly every case duplicates were aerated and afterwards the ammonia from one was determined by titration and from the other by nesslerisation.

In all the ammonia determinations the source of histaminase was a potent hog kidney powder, of which 50 mg. would inactivate 2 mg. histamine in 24 hours at 38°. For each determination 1 g. of this powder and 120 mg. histamine acid phosphate in 100 cc. phosphate buffer were employed. Initially the incubation was carried out in buffer solutions at p_H 7·2 but later two other hydrogen ion concentrations were used. The amount of residual histamine in each case was measured in the customary manner. In place of toluene, "merthiolate"[1] in 0·01 % concentration was used as preservative.

The first series was designed to measure the ammonia produced during the histamine-histaminase reaction under the conditions normally used by us: p_H 7·2, 24 hours' incubation at 38°. At first controls were flasks in which the enzyme had been inactivated by boiling and in these some ammonia was found. The difference between the ammonia content of the active enzyme flask and the boiled control was not of a simple molar nature and it was found that this type of control was not satisfactory. Although there is no histamine in the kidney powder, incubation of the powder in buffer without added histamine gives a demonstrable amount of ammonia, produced from the powder during the incubation, and appreciably greater than the amount found in a boiled control. Consequently similar mixtures of unheated powder and buffer with preservative were used in all subsequent work as controls. Table II, A gives results secured at p_H 7·2.

[1] The Eli Lilly Co.

Table II. *Ammonia formation.*

Histamine inactivated mg.	Total possible ammonia from histamine mg.	Ammonia found (mg.) Titration	Nessler	Average	Ammonia produced from histamine mg.	Ammonia found in % of theoretical
A. At p_H 7·2:						
2	0·90	0·61	0·67	0·64	0·29	32
0	0	0·32	0·37	0·35		
2	0·90	0·59	0·61	0·60	0·29	32
0	0	0·29	0·33	0·31		
3	1·35	0·79	0·78	0·79	0·46	34
0	0	0·31	0·35	0·33		
2	0·90	0·57	0·65	0·61	0·32	35
0	0	0·27	0·31	0·29		
4·0	1·80	0·90			0·56	31
0	0	0·34				
1·5	0·68		0·44		0·19	28
0	0		0·25			
					Average = 32	
B. At p_H 5·9:						
1·6	0·72	0·39	0·37	0·38	0·15	21
0	0	0·23	0·22	0·23		
1·6	0·72	0·33	0·29	0·31	0·14	19
0	0	0·17	0·16	0·17		
1·8	0·81	0·55	0·47	0·51	0·15	19
0	0	0·36	0·35	0·36		
1·4	0·63	0·29	0·27	0·28	0·12	19
0	0	0·17	0·15	0·16		
2·0	0·90	0·53	0·44	0·49	0·15	17
0	0	0·35	0·33	0·34		
1·6	0·72	0·34	0·31	0·33	0·14	19
0	0	0·19	0·18	0·19		
2·0	0·90	0·67	0·54	0·61	0·22	24
	0	0·40	0·38	0·39		
1·8	0·81	0·35	0·35	0·35	0·15	19
0	0	0·19	0·21	0·20		
2·0	0·90	0·64	0·59	0·62	0·20	22
0	0	0·42	0·42	0·42		
					Average = 20	
C. At p_H 8·8:						
3·6	1·62	0·70	0·65	0·68	0·47	29
0	0	0·21	0·21	0·21		
4·0	1·8	0·91			0·49	27
0	0	0·42				
4·0	1·8	0·74			0·53	29
0	0	0·21				
4·0	1·8	0·91			0·49	27
0	0	0·42				
2·0	0·90	0·40	0·41	0·41	0·23	26
0	0	0·17	0·19	0·18		
2·0	0·90	0·48	0·43	0·46	0·27	30
0	0	0·20	0·18	0·19		
3·5	1·61	0·65	0·66	0·66	0·46	29
0	0	0·19	0·21	0·20		
3·1	1·40	0·62	0·55	0·59	0·41	29
0	0	0·19	0·17	0·18		
					Average = 28	

The average amount of ammonia found was 32 % of the quantity which could be produced if all three nitrogen atoms in the histamine molecule had been liberated as ammonia. Under these conditions, then, only one nitrogen, probably from the side-chain, is liberated as ammonia.

It was imperative to make sure that the procedures used were giving reliable results. Aeration of ammonium sulphate solution and of kidney solution containing added ammonium sulphate showed that the analytical method was accurate. Moreover recovery of ammonium sulphate which had been added to digests before incubation indicated that ammonia was not lost during incubation. It is reasonable to conclude that the amount of ammonia measured is the total amount produced during the histamine-histaminase reaction under the experimental conditions.

Determinations were made of ammonia production at two other hydrogen ion concentrations, all other conditions being as similar as possible to those used at p_H 7·2. The results are given in Table II, B and C. As before, control experiments showed that ammonia was quantitatively recovered. While the ratio of ammonia produced to histamine inactivated at p_H 8·8 was slightly lower than at p_H 7·2 the difference is too small to be significant and again one molecule of ammonia was formed per molecule of histamine inactivated. At the other p_H, 5·9, this is obviously not true, the amount of ammonia being much less than at 7·2 and at 8·8. The explanation of this is not obvious at present. Since the amount of ammonia found is less than would be expected if all the inactivated histamine had been deaminised it is reasonable to conclude that a mechanism other than deaminisation is operative. Previous work in this laboratory had shown that under certain conditions there was a rupture of the iminazole ring. In the histamine-histaminase reaction both deaminisation and a change in the nucleus may take place and the relative intensities of these two processes may not be the same at different hydrogen ion concentrations.

The rate of ammonia formation in comparison with the rate of loss of physiological activity at p_H 7·2 was measured using a procedure similar to that previously described for velocity measurements. At definite time intervals portions were removed from the incubated mixture and measurements made of histamine and of ammonia present. The control again consisted of a kidney extract without added histamine and the ammonia from histamine was calculated as the difference between that of the histamine mixture and of the control. The results are given in Fig. 5. Calculations were made of the ammonia theoretically possible at each interval, assuming that one molecule had been produced for each molecule of histamine inactivated. Between the curve for these values and that for experimental results there is good agreement. At this p_H, then, the rate of ammonia production is in accordance with the velocity of histamine inactivation.

It seemed possible that urea might be formed during the reaction by a cleavage of the iminazole nucleus. Urea determinations in inactivated mixtures were made by the urease method and it was found that no compound which

could be decomposed by urease to give ammonia had been formed. Urea added to the solution was quantitatively determined by the method, indicating that the urease had not been inhibited by a constituent of the mixture. It may be that a urea derivative which could not be split by urease is formed but certainly urea itself is not produced during the histamine-histaminase reaction.

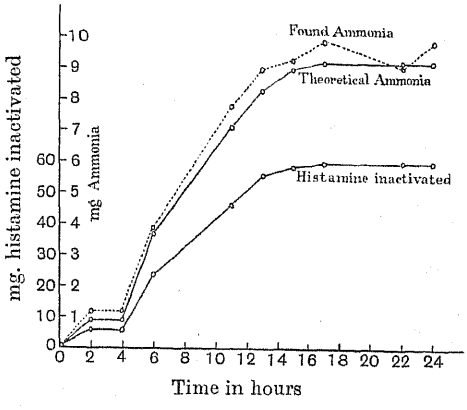


Fig. 5. Rate of ammonia formation.

II. Attempts to purify histaminase.

Since it is essential to prepare from kidney powder a solution containing the enzyme, conditions for increasing the yield on extraction have been studied. Water gives a potent extract, but the yield is greater when phosphate buffer at p_H 7·0–7·2 is used. Solutions thus secured are unstable but may be stabilised by the addition of glycerol, sufficient to give 15 % concentration. Extracts prepared at 38° are more potent than those at room temperature and these in turn are more potent than those secured by extraction in the cold. Solutions prepared by extracting at $p_H < 5$ are inactive and are not activated by neutralisation after extraction. A slightly increased yield is secured by extraction at p_H 8 but the alkaline solution filters very slowly. Larger yields are given by fairly dilute extraction, and we have found a suitable proportion to be 18 parts of liquid to one part of kidney powder. One such extraction at 38° gives about 60 % of the total potency of the powder, and a further 20 % results from a second extraction. The combined extracts may be concentrated *in vacuo* to one third the original volume without loss in activity, provided that the temperature of the bath surrounding the distillation flask be kept below 50°.

Three methods for the precipitation of histaminase have been investigated. If the concentrated, aqueous solution be poured slowly, with stirring, into a large volume of acetone at room temperature, a precipitate is secured which contains about 70 % of the total activity. This precipitate, after decantation of the acetone, dries quickly in air, and is relatively stable. If alcohol, ether, or mixtures of alcohol and ether are used in place of acetone the precipitate

produced is inactive. Precipitates which settle out from the original aqueous solution after adjustment to p_H 5 are also inactive. Four salts have been tried as precipitating agents. Sodium chloride in 25 % concentration at room temperature and sodium sulphate in various concentrations up to saturation at room temperature do not precipitate any active histaminase, despite the fact that the latter salt gives a heavy precipitate. When a solution is saturated with ammonium magnesium sulphate, histaminase is completely precipitated. This precipitate may be dried in a vacuum oven at temperatures below 50° without decrease in potency, and the dry powder is fairly stable, retaining its activity for at least a month.

The ammonium sulphate precipitate contains much sulphate, but a precipitate with low sulphate content is secured by using 80 to 90 % saturation. At this concentration of ammonium sulphate histaminase is completely precipitated. The conventional method for removing salt by dialysis results in a loss of about 70 % of the enzymic activity. A procedure developed by Brieger and Cohn [1893], and suggested to us by Dr P. J. Moloney, for the separation of tetanus antitoxin from ammonium sulphate has proved useful. This is based on differences in specific gravity. The salt-protein mixture is rubbed up with chloroform, more chloroform added and the whole centrifuged. Two solid layers separate: at the top proteins, and at the bottom the salt. This procedure gives good results with the histaminase precipitate. The top layer is poured on a hard filter-paper and allowed to dry in air. The resulting fine, tan-coloured powder contains over 70 % of the potency. Its activity is about ten times that of the original kidney powder, and it is relatively stable. While this procedure is an improvement on previous methods, it has not yet been possible to prepare histaminase in highly concentrated form.

III. Distribution of histaminase in the kidneys of various species.

It had previously been shown that, in the dog, histaminase was present in largest amounts in the kidney and in the intestinal mucosa. It seemed desirable to determine the variation in kidney histaminase among several species of animals. For this, where possible, normal animals were placed under ether anaesthesia and the kidneys removed. In some cases tissues were secured from abattoirs as soon as possible after the death of the animal. The human kidneys were autopsy specimens. In larger animals each kidney was tested separately while in smaller animals it was necessary to combine the kidneys from several animals to secure adequate amounts. After finely mincing the tissue, portions were weighed and transferred to 500 cc. Erlenmeyer flasks. In every case 5 g. of tissue were used for each determination. To each flask were added 100 cc. phosphate buffer, p_H 7·2, the required amount of histamine acid phosphate, and 15 cc. toluene. The mixture was incubated for 24 hours at 38° and the residual histamine determined by physiological assay. With each set controls were run, in which the enzyme had been inactivated by heating to boiling.

These controls acted as checks upon the total amount of histamine present, both added and contained in the tissue originally. The amount of histamine inactivated by a given amount of enzyme varies somewhat with the amount of histamine initially present. In each case determinations with varying amounts of histamine were made. The maximum inactivation was secured when 150 mg. of histamine acid phosphate (50 mg. histamine) were used with 5 g. kidney. The results in Table III are based on this proportion, and are averages of determinations made on four or more members of each species.

Table III. *Histamine inactivated by kidneys from various species of animals.*

Animal	Mg. histamine inactivated by 1 g. kidney
Rat	0
Guinea-pig	0
Rabbit	1·6
Dog	1·9
Cat	2·0
Ox	1·6
Hog	3·6
Horse	2·7
Sheep	3·2
Monkey	2·4
Human	0–1·6
Chicken	0

There is no apparent correlation between these results and available figures for histamine distribution in various species (Raxlen, unpublished data), or for sensitivity to histamine [Schmidt and Stähelin, 1929]. Among the individuals in any one species there is considerable variation, as shown in Table IV.

Table IV. *Kidney histaminase variation in members of a species*

Animal	Mg. histamine inactivated by 1 g. kidney	Average
Sheep	4·0, 2·8, 3·2, 4·0, 2·0, 3·2	3·2
Ox	1·2, 2·0, 1·2, 2·0, 1·2, 2·0	1·6
Hog	4·0, 2·8, 3·2, 3·2, 5·2, 3·2	3·6

As far as could be ascertained, the kidneys used in these distribution studies were normal. It would be interesting to investigate the histaminase content of kidneys in various pathological conditions. Preliminary experiments, using cats and dogs, were made in an attempt to alter the histaminase content of the kidney by extended administration of histamine subcutaneously, or by holding the animal in histamine shock. The results have been inconclusive. There has been no marked depletion in kidney histaminase after shock nor has it been found possible to increase kidney histaminase in dogs by giving histamine in small daily doses over a long period.

Summary.

Previous to the actual inactivation of histamine by histaminase there is a lag period, the length of which varies inversely with the enzyme concentration. The rate of the inactivation is logarithmic and, as judged from values secured for the velocity constant, the reaction is unimolecular. The inactivation

is accelerated in an atmosphere containing oxygen and is inhibited in an atmosphere deficient in oxygen. Small amounts of potassium cyanide not only prevent the initiation of the reaction but cause its cessation when added at an intermediate point. Sodium pyrophosphate has no inhibitory effect and the phosphate ion augments the reaction. Measurements of the ammonia production from histamine show that at p_H 7·2 and 8·8 histaminase causes the liberation of one nitrogen atom, presumably that of the side-chain, as ammonia. At p_H 5·9 less ammonia is produced. While attempts at purification have not as yet yielded a very concentrated preparation, information on a number of the properties of histaminase has been obtained. An investigation of kidney-histaminase in various species has shown that it is not demonstrable in the rat, guinea-pig and chicken, and is present in varying amounts in several mammals. Of the tissues so far investigated, hog kidney is the best source of histaminase and a method has been described for the preparation from this tissue of a stable powder which may be used in the study of histaminase.

This investigation has been carried out under the helpful direction of Prof. C. H. Best, whose advice has been greatly appreciated. It is a pleasure to acknowledge the technical assistance of Miss Neta Thompson. The preparation of kidney powder was made possible by the use of large-scale equipment in the Connaught Laboratories, kindly placed at our disposal by the Director, Dr J. G. FitzGerald.

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CLXIII. THE ELECTROLYTE DISTRIBUTION AND THE ACID-BASE EQUILIBRIUM IN THE SERUM IN CASES OF NEPHRITIS AND NEPHRITIC ACIDOSIS[1].

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We are presenting data obtained by the determination of the principal electrolytes of the blood in a series of cases of renal insufficiency with and without so-called renal acidosis. Various investigators, following Sellards [1914], have shown that if renal insufficiency is advanced the excretion in the urine of acid salts, particularly of phosphates and sulphates, is decreased. Marriott and Howland [1916] demonstrated the increase of phosphates in the blood in such cases. Denis [1921] was the first to show that inorganic sulphates might be increased in the serum in cases of renal insufficiency. Loeb and Benedict [1927] later observed a few cases of renal insufficiency in which the sulphate content of the serum was increased. More detailed studies of the inorganic sulphates in the serum in renal insufficiency have been reported by Wakefield [1929] and by Wakefield and Power [1930], Wakefield and Keith [1930], Wakefield *et al.* [1931]. Atchley and Benedict [1930] and Briggs [1932] recently have summarised the literature dealing with the acid-base equilibrium in nephritis. In their studies they reported values for the total base and for the anion distribution (chloride, carbonate, phosphate, protein, sulphate and ketone).

Our chief concern in this study was to establish the relation of inorganic sulphates to the various anions and cations in renal insufficiency and renal acidosis. Atchley and Benedict [1930] and Briggs [1932] determined sulphates but did not determine the various cations.

Methods of study.

The methods used for determination of sodium, potassium, calcium, magnesium, chloride, bicarbonate and phosphate were those described by Greene and Power [1931]. Sulphates were determined by the method of Wakefield and Power [1930], and lactic acid in tungstic acid filtrates by the method of

[1] Reported in part before the American Society for Clinical Investigation, Atlantic City, New Jersey, May 5, 1930.

Friedemann, Cotonio and Shaffer [1927]. In the present series we have determined the total base by summation of the values for the individual bases. Atchley and Benedict [1930] pointed out that the method of Stadie and Ross [1925] for the determination of total base "is a treacherous method, requiring constant use...to prevent errors. No method requires more vigilance to make it acceptable." We have found that with care the two methods give identical results, but in the present series the method of summation was used. Previous workers have used the formula of Van Slyke *et al.* [1928] for calculating the total base bound by protein. Greene and Power [1931] obtained a slightly larger value for the base bound by protein from their study of the equilibrium between serum and the *in vivo* dialysate. Both values are included in the accompanying tables.

Results.

The composition of the blood and serum in the ten cases studied is given in Table I. The electrolyte distribution and the acid-base equilibrium are shown in Table II.

The normal range for the total base in the serum is assumed to be 150 to 160 milliequiv./litre of serum. Atchley and Benedict [1930] reported 148 to 155 milliequiv.; we found 144 to 157 in a series of cases of ascites or hydrothorax of non-nephritic origin [Greene *et al.*, 1931]. Briggs [1932] reported values of 145 to 152 in cases of nephritis. In the present series the value for the total base ranged from 144·1 milliequiv. in case 9 to 167·5 milliequiv. in case 2 (Table II), with an average of 150·4 milliequiv. The difference between the total base contents of different samples of serum, as was to be expected, was due almost entirely to variations in the sodium. The changes in the potassium and magnesium were minimal, the calcium was less than 10 mg. in all cases and was reduced below 8 in cases 1, 3 and 5. A minimal value of 4·9 mg. was observed in case 3. In the majority of these cases the change was no greater than could be accounted for by either the increase in phosphates or the reduction in proteins, and there was fair agreement between the calcium actually determined and that calculated by the nomogram of Peters and Eiserson [1929]. Greenwald [1931] has pointed out the difficulties inherent in a mathematical formulation of the relationship between either the serum-protein or serum-phosphate and the calcium. Nevertheless, it may be accepted that in nephritis there is a tendency for the calcium content of the serum to vary directly with the proteins and inversely with the phosphates [Albright *et al.*, 1931; Farquharson and Tibbets, 1931; Peters and Van Slyke, 1931].

In three cases (1, 3 and 5) the calcium content of the serum was less than could be accounted for by the changes in protein or phosphates. The occurrence of renal rickets has been accepted by some as evidence of a primary disturbance in calcium metabolism associated with renal insufficiency. Greenwald [1918] found that intravenous injection of solutions of sodium sulphate will produce marked reduction in serum-calcium. The amounts of sulphates in the serum in nephritis are in no way comparable with those

Table I. *Composition of blood and serum in nephritis.*

Age years and sex	Urea mg./ 100 cc. whole blood	Creatinine mg./ 100 cc. whole blood	Protein g./ 100 cc. serum	Sodium mg./ 100 cc. serum	Potassium mg./ 100 cc. serum	Calcium mg./ 100 cc. serum	Magnesium mg./ 100 cc. serum	Chlorides NaCl mg./ 100 cc. serum	Carbon dioxide content volumes/ 100 cc. serum	Phosphates P mg./ 100 cc. serum	Sulphates SO_4 mg./ 100 cc. serum	Lactic acid mg./ 100 cc. serum	Diagnosis
38 F.	415	15·0	6·80	322	23·3	6·2	2·8	560	26·2	12·0	77·6	5·2	Hypertension, grade 4; renal arteriosclerosis; uraemia
35 M.	339	18·8	6·12	353	28·5	8·0	3·4	621	40·3	12·6	28·4	10·4	Renal arteriosclerosis; uraemia
19 M.	315	11·0	6·05	327	24·2	4·9	2·2	625	21·5	9·5	39·3	3·4	Chronic glomerulonephritis; renal dwarfism
22 M.	271	12·0	6·65	304	23·7	8·0	3·0	475	67·7	10·5	25·3	5·0	Chronic glomerulonephritis; uraemia
60 M.	219	7·0	6·35	325	22·0	6·5	2·4	638	36·7	8·3	24·6	5·4	Hyperprostate uremia; pyelonephritis
54 M.	151	8·1	5·45	308	25·2	8·3	3·1	582	50·9	5·3	19·6	6·1	Renal arteriosclerosis; uraemia
58 F.	115	5·0	6·85	316	21·9	9·6	3·0	637	41·7	5·4	12·0	4·5	Renal arteriosclerosis; uraemia
36 F.	95	8·1	6·95	305	22·9	9·1	3·0	608	43·8	5·3	16·2	6·3	Chronic glomerulonephritis
30 M.	10	1·0	4·54	309	19·3	8·2	2·8	581	58·3	3·8	6·5	12·8	Chronic glomerulonephritis with oedema
24 M.	23	1·3	4·80	317	20·3	8·4	2·0	596	64·2	3·9	3·5	4·3	Chronic lipoid nephrosis with oedema

Table II. *Electrolyte distribution of serum in nephritis: milliequiv./litre of serum.*

Case	Sodium	Potassium	Calcium	Magnesium	Total base	Chlorides	Bicarbonate	Phosphates	Sulphates	Lactic acid	Determined acids	Base less determined acids	Base proteinate: Calculated by formula I*	Base proteinate: Calculated by formula II	Total acids: Determined acids + B proteinate formula I	Total acids: Determined acids + B proteinate formula II	Total base less total acids as: Calculated by formula I*	Total base less total acids as: Calculated by formula II†
1	140·0	6·0	3·1	2·3	151·4	95·7	11·2	6·9	16·2	0·6	130·6	20·8	15·7	21·8	146·3	152·4	+ 5·1	− 1·0
2	153·4	7·3	4·0	2·8	167·5	106·1	17·0	7·3	5·9	1·1	137·4	30·1	14·4	19·3	151·8	156·7	+15·7	+10·8
3	142·1	6·4	2·5	1·8	152·8	117·1	9·1	5·5	8·2	0·4	140·3	12·5	14·3	19·1	154·6	159·4	− 1·8	− 6·6
4	132·2	6·1	4·0	2·5	144·8	81·4	29·0	6·1	5·2	0·6	122·3	22·5	15·7	21·0	138·0	143·3	+ 6·8	+ 1·5
5	141·3	5·6	3·3	2·0	152·2	109·1	15·5	4·8	5·1	0·6	135·1	17·1	15·0	20·1	150·1	155·2	+ 2·1	− 3·0
6	133·9	6·4	4·2	2·5	147·0	101·4	21·7	3·1	4·1	0·7	131·0	16·0	13·0	17·4	144·0	148·4	+ 3·0	− 1·4
7	137·3	5·6	4·8	2·5	150·2	108·8	17·3	3·1	2·5	0·5	132·2	18·0	16·2	21·6	148·4	153·8	+ 1·8	− 2·6
8	132·6	6·0	4·5	2·5	145·6	103·9	18·5	3·1	3·6	0·6	129·7	15·9	16·4	21·9	146·1	151·6	− 0·5	− 6·0
9	132·7	5·0	4·1	2·3	144·1	99·9	21·5	2·0	1·3	1·4	126·1	18·0	10·7	14·3	136·8	140·4	+ 7·3	+ 3·7
10	137·8	5·2	4·2	1·6	148·8	101·9	27·5	2·0	0·7	0·5	132·6	16·2	11·3	15·1	143·9	147·7	+ 4·9	+ 1·1

* Base bound by proteins as calculated by formula I [Van Slyke *et al.*, 1928].
† Base bound by proteins as calculated by formula II [Greene *et al.*, 1931].
\+ Represents excess of base.
− Represents excess of acid.

present in the experiments of Greenwald, but it is possible that retention of sulphates is one factor in the reduction of the serum-calcium in these cases.

The changes in the various anions are more striking than those in the various bases. Atchley and Benedict [1930] point out the difficulty of explaining the apparent loss of chlorides in many cases of nephritic acidosis. The chlorides were essentially normal in the majority of these cases. There was marked reduction in case 4 in which the chlorides fell to a level of 81·4 milliequiv. in consequence of prolonged vomiting. This case is of interest in that there was a compensatory increase in the bicarbonate to make up for the low values for chloride. Briggs [1932] likewise minimises the extent of the change in chlorides produced solely by impaired renal function. There was retention of sulphates and of phosphates to the extent of 10 milliequiv. or more in the first five cases (Table I). Phosphates and sulphates tend to be retained in the same cases, but apparently there is no necessary relationship between the degree of retention of either. The maximal value for the two was 23·1 milliequiv. (15 % of the total base). Under these conditions the part played by retention of phosphates and sulphates in the production of acidosis is manifest.

The data given in Table I for the lactic acid content of the serum indicate that there was no retention of this substance in the cases studied. On the contrary, the values seem to be somewhat lower than usual. According to Noshi [1929], the concentration of lactic acid in the serum is only slightly higher than that in whole blood. Recent determinations of the lactic acid in whole blood, by the same methods as were used in the present study, have been made by Campbell and Maltby [1928] in a series of thirty-five cases, in which metabolic abnormalities were not present. The lactic acid content of the blood of these fasting subjects averaged 14·1 mg./100 cc.; in thirty-one cases (88·5 %) it fell within the range 10–17 mg./100 cc. In a less extensive series of determinations on fourteen adult subjects without diabetes, Koster *et al.* [1930] obtained somewhat higher values, averaging 21·3 mg./100 cc. Bishop [1931] determined the lactic acid in the blood of five normal subjects by means of a slight modification of the Friedemann, Cotonio and Shaffer [1927] method, with results ranging from 10·6 to 16·0 mg. and averaging 13·2 mg./100 cc. Wakefield and Greene [1930] found values of 7–26 mg. in a series of cases of various types of hepatic disease.

In comparison with these data, it is evident that the values for the lactic acid content of the serum are markedly lower in the present series of cases. The lactic acid in the blood tends to be low in conditions of experimental acidosis [Anrep and Cannan, 1923] and conversely to increase in alkalosis [Frank, Leiser and Weisz, 1930]. In the cases studied, however, there was no apparent relationship between the amount of lactic acid present and the degree of acidosis, as indicated by the carbon dioxide content of the serum. It may be therefore that the low values for the lactic acid are consequent on the prolonged rest in bed undergone by these patients rather than on other factors.

Jervell [1929] studied the lactic acid content of the blood in a series of cases of acute nephritis by means of a method apparently much less specific than the one we used, but found no evidence for retention of lactic acid. Neither did he find the decreased values such as we have encountered in most of the cases under consideration here. In the latter, lactic acid plays a negligible part so far as the acid-base balance is concerned. Atchley and Benedict [1930] have shown the same to be true of the different ketone substances.

When the sum of the determined acids is subtracted from the value for total base, there is an undetermined fraction of 12·5–30·1 milliequiv. Almost all, if not the entire amount, of this undetermined fraction represents base bound by protein. If the formula of Van Slyke *et al.* [1928], which was determined by the titration of the purified proteins of horse-serum, is used for calculation of the latter, and this value is then added to the sum of the determined acids, there remain, on the average, 4·4 milliequiv. of base unaccounted for, and which presumably indicate the presence of undetermined organic acids. If the figures of Greene and Power [1931], which were calculated from the composition of the dialysate of dog's blood obtained *in vivo*, and from the comparative composition of serum and transudates of patients in hospital, are used for the calculation of the base bound by protein, and this value is added to the sum of the determined acids, the resulting figures for the total anions, on the average, are larger by 0·4 milliequiv. than the values for total base. The differences between the values for the total base and the sum of the acids in either case are within the analytical error of the method for determination of total base.

The values for the base-binding power of the serum-proteins were determined by Van Slyke *et al.* [1928] by the titration of dialysed protein solutions with sodium bicarbonate. The values which they obtained agree well with the values found by Greene and Power [1931] for the sodium bound to protein. Greene and Power also found that in the serum *in vivo*, potassium, calcium and magnesium combine with small amounts of protein and so increase the value for the total base bound by protein. In the present instance the base bound by protein was calculated on the assumption that the serum contained a normal albumin-globulin ratio and that the p_H was 7·35. Disturbances in both p_H and in the albumin-globulin ratio are to be expected in nephritis with consequent shifts in the amount of base bound by protein, although these changes presumably are slight. Regardless of the particular figures chosen, the fact remains that when the chlorides, bicarbonate, phosphate, sulphate and lactate in serum are determined, protein will account for practically all the remaining base. A small amount of unidentified organic acids may be present, but, if so, they are apparently negligible in quantity.

Discussion.

The literature dealing with changes in individual electrolytes in the serum in cases of nephritis and nephritic acidosis has been reviewed in detail by

Peters and Van Slyke [1931], and need not be discussed again here. In general, the changes observed in the present series of cases accord with previous observations. In some cases there was reduction in total base, but this change was neither as uniformly observed nor as marked in degree as suggested by previous investigators. When the total base was reduced, the change was due almost entirely to changes in the amount of sodium. Calcium was reduced in several cases, the reduction being associated in part with reduction in the serum-proteins, and perhaps in part with the retention of phosphates and sulphates. There was no evidence for the accumulation in the blood of significant amounts of undetermined organic acids. In the majority of instances, the chlorides were normal, but the importance of vomiting in causing a reduction in the serum-chloride should be emphasised. There is an accumulation of phosphates and sulphates due presumably to renal insufficiency. This retention of inorganic acids plays an appreciable part in the production of acidosis. The changes in bicarbonate depend on the balance between the opposing effects of loss of chloride on the one hand and the loss of base and retention of phosphates and sulphates on the other. Usually, the bicarbonate is decreased.

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CLXIV. THE PRECIPITATION AND PROTECTION OF VANADIC ACID BY SERUM-PROTEINS.

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Zsigmondy [1901] has shown that proteins inhibit the precipitation of red colloidal gold which occurs when salts are added. This effect is however at a minimum at the isoelectric point of the protein. Wright and Kermack [1923, 1, 2] were able to show that proteins on the acid side of the isoelectric point, and in dilute solution, caused precipitation of colloidal gum benzoin. Hopkins, Kermack and Spragg [1931, 1] obtained similar results with gum benzoin and with colloidal gold, which are positive lyophobic colloids. The reactions between acid solutions of protein and negative lyophilic colloids have not previously been studied. While the lyophobic sols depend upon their electric charge alone for their stability, the lyophilic proteins have both an electric charge and an affinity for the dispersion medium. In the neutral and alkaline solutions studied by Zsigmondy, the proteins bear a negative charge like that of the gold particles, but in acid solution the charge on the protein particles becomes positive and being opposite to that of the gold particles, causes neutralisation and precipitation. Although this "electrostatic effect" (as Hopkins, Kermack and Spragg [1931, 2] have called it) changes with p_H, the "proper protective effect" of the protein remains, and so some proteins in higher concentration may still protect lyophobic solutions, even at their isoelectric point. There is too, an intermediate effect of sensitisation, which has been investigated by Pauli and collaborators [see Pauli, 1930]. The reaction between ferric hydroxide sol and proteins described as sensitisation by Reitstöter [1920] is really a case of mutual precipitation of oppositely charged colloids, comparable to some extent with the precipitation of gold sol with acid solutions of proteins observed by Hopkins, Kermack and Spragg, but more nearly comparable with the phenomena described below in which two oppositely charged lyophilic colloids are concerned. The term sensitisation should be limited to those cases where the protein increases the flocculability of a sol. In the present work most of the experiments were carried out in the presence of a low constant concentration of sodium acetate so that sensitisation effects were not sought for and none was observed. This was essential because of the impossibility of preparing buffered solutions of the acids under consideration in the absence of salts and of dissolving serum-euglobulin without salt being

present. The theoretical aspect of the experiments does not suffer from this omission as it will be seen that in acid solution there is always a tendency to precipitation with added protein even without the addition of any salt.

Many hydroxy-acids have a strong tendency to form poly-acids in acid solution. Such acid radicals may be colloidal, and Graham [1864] prepared and investigated colloidal tungstic acid. Silicic acid, metastannic acid and antimonic acid are familiar examples. Britton and German [1930] found that sodium molybdate on acidification to p_H 4·8 begins to form polymolybdic acid in which the acid $H_{10}Mo_{12}O_{46}$ has been identified. Arsenic acid, like phosphoric acid, does not form very complex acids. Antimonic and bismuthic acids form complex colloidal solutions but are unsuitable for study in connection with proteins because they give precipitates on dilution or on change of acidity of their solutions. Vanadium alone of the common elements of the fifth group forms stable poly-acids. Molybdic, tungstic and vanadic acids in addition to combining with themselves to form complex acids all combine with each other.

Düllberg [1903] investigated vanadates and vanadic acid and found that on acidification the simple vanadates formed hexavanadic acid, $H_4V_6O_{17}$, which gave tervalent ions $(HV_6O_{17})'''$. Such an ion would have a molecular weight of almost 600 and it is possible that more complex molecules occur.

The acid solutions of vanadic, molybdic and tungstic acids are negatively charged hydrated semi-colloids. They are quite stable, not being precipitated by salts in any concentration, but precipitated by equivalent amounts of a positive colloid such as $Fe(OH)_3$, but not if excess of the acid is present. In this work the reactions between these positively charged complex acids and acid solutions of proteins with negative charges are studied.

Experimental.

Solutions of the poly-acids were made by mixing decinormal solutions of salts of the acids with decinormal acetic acid in various proportions in order to give the requisite p_H. The solutions were then diluted so that each contained the same amount of the acid radical under consideration. In this way, solutions of various poly-acids in acetate buffer solutions were easily made. A similar technique was used by Bendien [1931] for making solutions of vanadic acids, for the purpose of a "diagnostic test for cancer," but in his case the vanadium present in each solution varied in addition to the hydrogen and sodium ion concentrations. Such solutions would be unsuitable for experimental purposes. The various solutions used were prepared as follows.

Vanadic acid. Na_3VO_4 solution (12·2 g. sodium vanadate (Kahlbaum) per litre) was mixed with varying amounts of $N/10$ acetic acid to give solutions varying in p_H from 3·2 to 5·4. Twenty solutions each with different proportions of acid and salt and different p_H were made. Each was then diluted so as to have the same concentration of vanadium (1 gram-atom V per 300 litres).

Molybdic acid and tungstic acid. *M*/70 ammonium molybdate (17·6 g. $(NH_4)Mo_7O_{24}$, $4H_2O$) and *M*/10 sodium tungstate (33·0 g. Na_2WO_4 per litre) were mixed with acetic acid. Ten solutions varying in p_H from 3·25 to 5·5 were made, each containing 1 gram-atom molybdenum or tungsten respectively per 300 litres.

The proteins were separated from normal human sera by dialysis and precipitation. The serum was dialysed in closed collodion sacs which were continuously raised and lowered gently in a vessel through which distilled water at a temperature below 8° was flowing at a rate of 700–800 cc. per hour.

The movement of the collodion sacs was so arranged that a bubble of air passed through the protein solution from end to end of the collodion sac each time it was raised or lowered. By this means the inside surface of the collodion was continually swept and dialysis was much accelerated. After the salts were removed from the serum in this way (2 to 3 days), the serum was centrifuged to remove the precipitated euglobulin. The clear liquid was then saturated with carbon dioxide in the cold when most of the pseudoglobulin was precipitated, and was removed by means of the centrifuge. The remaining solution contained the albumin possibly contaminated with a little pseudoglobulin, but the contamination is probably less significant than the change brought about by precipitation with strong ammonium sulphate solution [see Svedberg, 1931]. The albumin solution was used as obtained. The euglobulin precipitate was washed with distilled water and then dissolved in *M*/10 sodium acetate, in which it dissolved with difficulty, and only weak solutions could be prepared. The protein concentrations of the solutions used were determined by accurate estimation of the refractive index in a Zeiss interferometer and calculated by means of the data obtained by Adair and Robinson [1930].

The method of observing precipitation was similar to that of Hopkins, Kermack and Spragg [1931, 1], except that the ratio of volumes was that adopted by Bendien [1931]. Protein solutions made up to 0·4 cc. with water in each case were placed in a small tube and 2 cc. of the poly-acid solution were added with shaking. The tubes were allowed to stand for 24 hours and the formation or otherwise of a precipitate was then observed. Slight turbidity unaccompanied by any settling was not considered to be precipitation. The results are expressed graphically after the manner used by Hopkins, Kermack and Spragg [1931, 1]. The lines in the figures represent the boundary between precipitation and protection; points are marked at the minimum concentration of protein where it appeared that protection had occurred.

Albumin.

The results obtained with vanadic acid and serum-albumin are shown in Fig. 1. It will be seen that albumin is able to inhibit protection if it is present in sufficient concentration in solutions more acid than p_H 4·0. This effect is similar to that obtained by Hopkins, Kermack and Spragg with colloidal gold, in which the protective action was minimum about the isoelectric point. In

the experiments with colloidal gold much lower concentrations of albumin give protection than in the present experiments. This difference is largely due to difference in concentration of the sols used. (The gold sol used contained 1 gram-atom Au in 3000 litres.) The point of minimum protection with vanadic acid appears to occur at a p_H slightly more acid than with gold sol. The stable sol which is produced with excess albumin and vanadic acid was shown to be positively charged by means of cataphoretic experiments. Two platinum electrodes connected to a 6-volt battery were placed in a U-tube containing such a sol. After the current had been passing for an hour the yellow colour of the vanadic acid-protein complex had disappeared from the liquid surrounding the positive electrode and was deposited round the negative electrode. A similar experiment with vanadic acid in the absence of protein showed the vanadic acid to be negatively charged as would be expected from the ionisation of $H_3(HV_6O_{17})$.

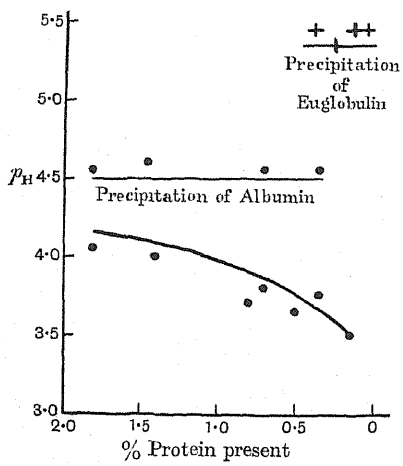


Fig. 1. Precipitation and protection of vanadic acid.

• Albumin
+ Euglobulin

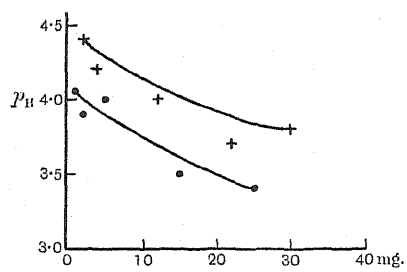


Fig. 2. Globulin in mg. held in solution by 100 mg. albumin in vanadic acid solution.

+ Pseudoglobulin (mg.)
• Euglobin (mg.)

Euglobulin.

The effect on euglobulin is however different. In all concentrations used euglobulin was precipitated in solutions more acid than p_H 5·2. Trials were made with $N/1000$ HCl present, giving a final p_H of 3·0, but precipitation still occurred. Experiments with half and quarter concentrations of vanadium with the maximum amount of globulin (0·05 %) also gave precipitates. Solutions more alkaline than p_H 5·4 were not precipitated at any concentration tried.

Pseudoglobulin.

The effect obtained with pseudoglobulin was different from that with albumin. Precipitation occurred at all hydrogen ion concentrations above p_H 5·0. Increase of concentration did not result in protection in any case,

so that pseudoglobulin is like euglobulin in its reaction to vanadic, tungstic and molybdic acids. It was also found that pseudoglobulin added to albumin which was itself protected caused precipitation. Albumin could however protect pseudoglobulin and the amount protected by a definite amount of albumin was practically the same as the amount of euglobulin protected, as can be seen from Fig. 2. The experiments described with albumin, euglobulin and pseudoglobulin and vanadic acid were repeated with tungstic and molybdic acids, and exactly the same results obtained. The globulin always gave a precipitate if the p_H were between 3·0 and 5·0. Increase in concentration of globulin did not give protection. On the other hand excess of albumin caused protection as it did with vanadic acid. The experiments were also repeated with fresh horse serum-proteins in place of human serum-proteins and the same results obtained. A preparation of commercial horse serum sterilised with chloroform gave albumin with little protective action. On the other hand a preparation of crystalline horse serum-albumin prepared in 1914, for which I am indebted to Dr H. Chick, had protective action identical with that of normal human serum-albumin. The addition of salts which might increase the charge on globulin and thus increase its protective action was tried. Globulin solutions with small additions of sodium chloride, barium acetate, aluminium chloride and sodium citrate were treated with vanadic acid in the usual way. In all cases precipitation occurred.

Mixtures of proteins.

When mixtures of albumin and euglobulin or pseudoglobulin were used precipitation occurred unless albumin were present in considerable excess. The amount of globulin which albumin could hold in solution varied with the p_H as can be seen in Fig. 2. The precipitation that occurs when there is sufficient albumin present to protect itself but not the globulin, involves all the vanadic acid, but not all the protein. A yellow precipitate settles, leaving a clear liquid above, which contains protein. The protein remaining in solution is not precipitated by half-saturated ammonium sulphate and must therefore be albumin.

Some experiments were also carried out with vanadate solution made in the concentrations described by Bendien. Similar results were obtained. Albumin in sufficient concentration inhibited precipitation and globulin was always precipitated. If a little globulin was added to a normal human serum previously giving a precipitate in Bendien's solution No. 8, precipitation could be produced with his solution 7, 6, 5 or 4, according to the amount added, *e.g.* 0·2 cc. serum containing 5·2 % albumin and 2·7 % globulin just gave a precipitate with 2 cc. Bendien solution No. 8; with the addition of 0·04 cc. of 0·38 % serum-globulin, precipitation occurred in tube 7, with 0·10 cc. in tube 6, with 0·14 cc. in tube 5, and with 0·20 cc. in tube 4.

Bendien [1931, p. 27] says that increased globulin in serum and the precipitation are not parallel. There would however appear to be no doubt that

changes in concentration are the main cause of differences in precipitation. Another cause of such differences is the total protein content—if the albumin content were sufficiently low precipitation would occur with all vanadic acid solutions even without globulin being present. Still another cause of differences in precipitation lies in differences in the alkali reserve of sera. It is possible for the alkali reserve of serum to vary from 25 to 75 volumes CO_2 per 100 volumes serum, *i.e.* from 0·02 *N* to 0·06 *N* alkali, a possible deviation of 0·04 *N*. Now the difference in acid content of the Bendien solutions (1 to 12) is 0·05 cc. *N*/10 acid per 10 cc., *i.e.* 0·0005 *N*. In the test, serum is diluted 10 times with the vanadic acid, so that the maximum differences in alkali reserve of serum can produce considerable differences in the final acidity and so in the precipitation. The Bendien test gives no real insight into the three factors which are involved, namely, albumin-globulin ratio, total protein content and alkali reserve, but merely indicates whether serum is normal or not.

Discussion.

It can be seen from these experiments that solutions of vanadic acid of varying acidity could be used as a rough guide in determining the ratio of albumin and globulin in a solution such as serum. With increase of acidity more globulin can be held in solution and the p_H at which precipitation occurred would indicate the ratio in which the two proteins were present. It is to this fact that the "diagnostic test for cancer" devised by Bendien [1931] is due, although this author did not appear to be aware of the true nature of his test.

The results of the experiments on the separate proteins can be explained mainly by means of their electric charge and their stability as lyophilic colloids. The proper protective effect, as distinct from the electrostatic effect, in the present experiments, however, appears small, being probably of the same order as the protective action of polysaccharides, because the negative colloid is itself lyophilic and hydrated. Hardy's [1899] original work showed that the main cause of stability of protein solutions is their electric charge; when this charge is removed, as at the isoelectric point, the solution becomes unstable. Kruyt and De Jong [1922] have been able to show that with increased charge there is an increase in the hydration, with which is bound up the proper protective effect. It is however in the electrostatic effect of the protein molecule that an explanation of the results described above is to be found.

If the charge on the protein molecules added (*i.e.* the total charge present, which is the product of the charge per molecule and the protein concentration) is small compared with that on the vanadic acid present then the protein charge is neutralised and precipitation occurs. If the charge on the protein is great enough then it can itself neutralise the vanadic acid charge and if any surplus electrostatic charge remains, precipitation need not occur. Although a stable sol is produced with excess of protein over vanadic acid, the

same does not occur with large excesses of vanadic acid. Both colloids are lyophilic and the difference in effect must be due to difference in size of the particles. The serum-albumin particles are probably 50 times as heavy as those of vanadic acid and for that reason cannot be held in solution by vanadic acid.

The difference in action of globulin and albumin could be attributed to difference in the respective charges. It has been shown [Cohn, 1925] that globulin has relatively few groups capable of ionisation on the acid side of the isoelectric point. The difference between the two proteins in this respect is really very considerable. About the isoelectric point serum-albumin requires 28×10^{-5} mols acid per g. protein to change the acidity by 1 unit of p_H. Serum-globulin under the same conditions requires but 5×10^{-5} mols acid to produce the same change in acidity. The charge on globulin in acid solution will probably therefore be about one-sixth of that present on serum-albumin under similar conditions. It will therefore have but a small charge in solution and this is neutralised by the positive vanadic acid. The charge on the globulin is not great enough to reverse the charge of the poly-acid. This effect is also helped because, owing to the low solubility, it is not possible to use concentrated solutions of globulins.

This however leaves the behaviour of the mixed proteins unexplained. The globulin molecules must have some negative charge and this charge should help the albumin to protect the solution against precipitation. In all experiments in acid solution however antagonism occurs. The effect can only be explained by some specific precipitation of euglobulin by these poly-acids. This precipitation may be related to the fact that globulin is soluble in salt solutions, but not soluble in the absence of salts. There is possibly some definite compound formed between euglobulin and salt and possibly complex acids, *e.g.* vanadic acid, combine in the same way giving an insoluble derivative.

Vanadic acid precipitates serum-globulin and this precipitation may be inhibited by serum-albumin as all precipitations are inhibited by the protective action of such a colloid.

Summary.

1. Vanadic, molybdic and tungstic acids form negative lyophilic semi-colloidal solutions.

2. Different serum-proteins react with such solutions in different ways. Serum-albumin is precipitated in low concentrations, but in higher concentrations there is no precipitation, protection occurs and a stable positive colloid is formed. Serum-globulin however causes precipitation in all available concentrations between p_H 3·0 and 5·3.

3. If euglobulin is added to an amount of albumin great enough to cause protection when a poly-acid is added alone, precipitation will occur. If sufficient excess of albumin is added the globulin will be held in solution. The

amount of globulin which a definite amount of albumin can protect increases with increase in acidity of the solution.

I wish to express my thanks to Prof. E. L. Kennaway for his interest in this work and to Prof. E. C. Dodds for the loan of the interferometer used in the experiments.

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CLXV. VAN SLYKE'S METHOD OF DETERMINATION OF ACETONE BODIES APPLIED TO SMALL VOLUMES OF BLOOD AND URINE.

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In the method described below the proportions of the various reacting substances have been adjusted with the help of data given by Van Slyke [1917; Van Slyke and Fitz, 1917], and the strengths of solutions have been so arranged that the volumes are easily and accurately measured. The mercury-acetone precipitate is titrated, minor improvements being made in the technique, and a table is supplied to facilitate calculations. For a single estimation 0·5 cc. of blood or 1 cc. of urine is needed. It is possible therefore to make determinations on capillary blood obtained by skin puncture.

The writer has made parallel determinations with Van Slyke's original technique and the micro-technique described below on diabetic blood and urine, on known aqueous solutions of pure β-hydroxybutyric acid and acetone, and on normal blood to which known amounts of β-hydroxybutyric acid and acetone had been added. He finds that if the acetone body content is of the order of that found in severe ketosis (0·1 % reckoned as acetone), the micro-technique gives fully as accurate results as the original gravimetric technique of Van Slyke. In lesser grades of ketosis, the micro-technique is less accurate than the original technique, but it gives results of value in clinical work where only relatively gross variations in the content of acetone bodies are of significance. It gives an approximate measure of the acetone-body content of blood and urine even in health.

Apparatus and reagents.

The blood or urine is measured with an accurate Ostwald pipette. A reflux condenser with a straight inner tube of 8 mm. diameter is required for the oxidation. For the titration of the mercury-acetone precipitate, a 5 cc. micro-burette with a fine tip so that each drop is 0·02 cc. or less, and a dark blue tile are needed.

The acid mercuric sulphate solution is prepared by dissolving 45·6 g. of pure red oxide of mercury in one litre of 2·5 N sulphuric acid. The $M/20$ mercuric chloride solution is made by dissolving 13·576 g. of the A.R. salt in

water and diluting to 1 litre; standardisation by the sulphide method as described by Van Slyke is unnecessary. The 3·3 % potassium iodide solution is checked against the $M/20$ mercuric chloride solution each time a test is made (see later).

With every new batch of reagents a blank determination must be made, using water instead of blood or urine, to test the purity of the chemicals.

Removal of interfering substances.

Proteins *etc.* are removed from blood as follows. 0·5 cc. of whole blood is laked in 20 cc. of distilled water in a 25 cc. volumetric flask. 3 cc. of the acid mercuric sulphate solution are added, the volume is made up to 25 cc., and the flask is inverted several times to mix. After standing for 15 minutes the flask is again inverted and the contents are filtered.

Glucose[1] and other interfering substances are removed from urine as follows. In a stoppered graduated tube or cylinder 4 cc. of water, 1 cc. of urine, 2 cc. of 20 % copper sulphate, and 2 cc. of 10 % calcium hydroxide suspension are mixed. After a few minutes the reaction is tested with litmus, and if it is still acid, more of the calcium hydroxide suspension is added drop by drop until the reaction is alkaline. The mixture is then made up to 10 cc., allowed to stand for 30 minutes and filtered.

Oxidation of β-hydroxybutyric acid and separation of the mercury-acetone compound.

In a 100 cc. conical flask are placed 20 cc. of blood-filtrate, 3 cc. of $14N$ sulphuric acid, and 3 cc. of the acid mercuric sulphate solution, or 14 cc. of water, 4 cc. of urine-filtrate, 3 cc. of $14N$ sulphuric acid, and 5 cc. of the acid mercuric sulphate solution.

The flask is connected with the reflux condenser and the flame turned on. When the contents have been brought to the boil, 2 cc. of 2 % potassium dichromate are run down the condensing tube. Gentle ebullition is maintained for exactly 30 minutes.

After thorough cooling, the contents of the flask are filtered through a 9 cm. retentive paper (*e.g.* Whatman No. 42) without suction, and the precipitate is washed free from the reagents with water. Five washings usually suffice. The precipitate tends to float to the surface, wherefore a funnel should be selected which is a little smaller than the paper. (An alundum crucible may be used for the filtration.)

Titration of the mercury in the mercury-acetone compound.

Into a beaker of about 10 cm. diameter, 5 cc. of N hydrochloric acid are measured. The filter-paper is unfolded and dropped into the acid. The beaker is heated gently until the precipitate has dissolved, and then cooled to room

[1] The urine must be diluted if it contains more than 8 % of glucose.

temperature. 2 cc. of 40 % sodium acetate, one or two drops of 10 % sodium sulphite and 5 cc. of 3·3 % potassium iodide are added in the above order.

The excess of potassium iodide is determined by titration with $M/20$ mercuric chloride, the latter being added drop by drop until, when viewed against a dark blue tile, a reddish precipitate persists. The filter-paper may be lifted out just before the end-point is reached.

The sulphite is added to reduce any chromate derived from the mercury-acetone compound. Otherwise iodine is liberated from iodide by the chromate, thus introducing an error of about 7 %.

A blank titration of the potassium iodide solution against the $M/20$ mercuric chloride solution must be made each time an estimation of acetone bodies is undertaken. The same pipette should be used for measuring the 5 cc. of KI solution in both blank and test titrations. The blank titration is performed as follows. In a beaker are placed 5 cc. of N HCl, 2 cc. of 40 % sodium acetate, one or two drops of 10 % sodium sulphite (as in the test titration), and 5 cc. of 3·3 % potassium iodide in the order given. The $M/20$ mercuric chloride is run in until the red precipitate persists.

It is important that the potassium iodide should be checked against the $M/20$ mercuric chloride in the above way, because the end-point is affected by the p_H of the reaction mixture, slightly more $HgCl_2$ being required for the titration of a given volume of KI solution with increasing acidity. Using 3·3 % KI and the above technique, about 4·8 cc. of $M/20$ $HgCl_2$ are required to complete the blank titration.

Differential estimation of acetone bodies.

The above description applies when all the acetone bodies are to be estimated together.

For acetone *plus* acetoacetic acid, the same procedure is adopted except that the amount of $14N$ sulphuric acid is reduced to 2 cc. and the potassium dichromate is omitted. The filtrate from the mercury-acetone precipitate is preserved separately from the washings for the subsequent determination of β-hydroxybutyric acid.

For this purpose, the filtrate is measured in a cylinder and transferred to a 100 cc. conical flask. The filter-flask and cylinder are then washed out with water sufficient to make a total of 25 cc., and the washings are also transferred to the conical flask. After adding 1 cc. of $14N$ sulphuric acid, the conical flask is fitted to the condenser, and the contents are heated to boiling-point. The subsequent treatment, including the addition of the 2 cc. of 2 % potassium dichromate, is exactly as described for the determination of total acetone bodies.

This procedure is necessary for blood of which, moreover, the quantity available is often too limited to allow of a separate determination of β-hydroxybutyric acid. It may of course also be applied to urine. A separate estima-

tion of β-hydroxybutyric acid, after acidification and boiling off the volatile acetone bodies, is however more satisfactory. The procedure is as follows.

To 14 cc. of water and 4 cc. of urine-filtrate, 1 cc. of 14*N* sulphuric acid is added. The mixture is boiled for 10 minutes and cooled. The volume is then readjusted to the original by the addition of water. The remaining 2 cc. of 14*N* sulphuric acid and 5 cc. of acid $HgSO_4$ are then added, and the determination is completed as described for total acetone bodies.

Calculation.

In the blank titration the 5 cc. of KI solution are balanced by the added *M*/20 $HgCl_2$. In the test titration a portion of the 5 cc. of KI solution has combined with the mercuric chloride formed by solution in hydrochloric acid of the mercury contained in the mercury-acetone precipitate: the rest of the 5 cc. of KI solution is balanced by the *M*/20 $HgCl_2$ required to complete the titration. Therefore the difference between the blank titration and the test titration (*i.e.* the *M*/20 $HgCl_2$ deficiency) measures the quantity of acetone bodies in the amount of blood- or urine-filtrate employed.

Acetone. The mercury-acetone compound contains 77 % mercury and therefore 1 cc. of *M*/20 $HgCl_2$ deficiency corresponds to 13 mg. of the mercury-acetone precipitate (*i.e.* 10 mg. mercury).

It is found that 1 mg. of acetone gives 20 mg. of precipitate, *i.e.* each mg. of precipitate corresponds to 0·05 mg. acetone.

From this it follows that 1 cc. of $HgCl_2$ deficiency represents 13 × 0·05 mg. of acetone; using a blood-sample of 0·4 cc. this means an acetone concentration of 13 × 0·05 × 250 or **162·5** mg./100 cc.

β-Hydroxybutyric acid. In the case of β-hydroxybutyric acid it is found that 1 mg. of the acid yields 6·7 mg. of precipitate.

But 104 g. (1 g.-mol.) of β-hydroxybutyric acid should yield 58 g. (1 g.-mol.) of acetone.

Whence 1 mg. of β-hydroxybutyric acid should yield $\frac{58}{104} \times 20$ mg. of precipitate.

This means that under the conditions of oxidation employed the yield of acetone from β-hydroxybutyric acid is $\frac{6{\cdot}7 \times 104}{58 \times 20} \times 100$ or 60 % of the theoretical; *i.e.* 1 g.-mol. of β-hydroxybutyric acid yields 0·6 g.-mol. of acetone.

Expressed numerically:

104 g. β-hydroxybutyric acid yield 0·6 × 58 g. acetone.

∴ 1 mg. acetone produced corresponds with $\frac{104}{0{\cdot}6 \times 58}$ mg. β-hydroxybutyric acid but 1 mg. acetone produced corresponds with 20 mg. ppt. and ∴ with $\frac{20}{13}$ cc. $HgCl_2$ deficit.

∴ 1 cc. $HgCl_2$ deficit represents $\frac{13}{20} \times \frac{104}{0{\cdot}6 \times 58}$ or 1·942 mg. β-hydroxybutric acid in the sample, *i.e.* 486 mg. β-hydroxybutyric acid per 100 cc. blood if the sample is 0·4 cc.

Recalculating as acetone this figure becomes $486 \times \frac{58}{104}$ or **270·8** mg./100 cc.

Total acetone substances. For the purpose of this calculation it is assumed that the mixture being analysed contains β-hydroxybutyric acid and acetone in the molecular ratio 3 : 1.

Now 3 g.-mols. of β-hydroxybutyric acid will yield 3 × 0·6 or 1·8 g.-mols. of acetone; *i.e.* 4 g.-mols. of the mixture will yield 1·8 + 1 or 2·8 g.-mols. of acetone.

Expressed numerically:

$$(3 \times 104 + 58) \text{ g. mixture yield } 2{\cdot}8 \times 58 \text{ g. acetone,}$$

whence 1 mg. of acetone produced represents $\frac{370}{2{\cdot}8 \times 58}$ mg. mixture.

$\therefore$ 1 cc. of $HgCl_2$ deficit represents $\frac{13}{20} \times \frac{370}{2{\cdot}8 \times 58} = 1{\cdot}481$ mg. mixture in sample, or 1 cc. of $HgCl_2$ deficit represents $1{\cdot}481 \times 250 = 370$ mg. mixture/100 cc. blood (the sample being 0·4 cc.).

In order to convert to acetone the last figure must be multiplied by $\frac{4 \times 58}{370}$, giving **232·1** mg./100 cc. blood as acetone. Figures worked out on this basis for $HgCl_2$ deficits up to 1 cc. using 0·4 cc. samples are given in Table I.

Table I. *Relationship between the M/20 $HgCl_2$ deficiency and concentration of acetone bodies in blood or urine.*

(Sample = 0·4 cc.)

cc. *M*/20 $HgCl_2$ deficiency	Total acetone bodies as mg. of acetone per 100 cc.					Acetone and acetoacetic acid as mg. of acetone per 100 cc.					β-Hydroxybutyric acid as mg. of acetone per 100 cc.				
	·00	·02	·04	·06	·08	·00	·02	·04	·06	·08	·00	·02	·04	·06	·08
0·00	0	5	9	14	19	0	3	7	10	13	0	5	11	16	22
0·10	23	28	32	37	42	16	20	23	26	29	27	32	38	43	49
0·20	46	51	56	60	65	33	36	39	42	46	54	60	65	70	76
0·30	70	74	79	84	88	49	52	55	59	62	81	87	92	97	103
0·40	93	97	102	106	111	65	68	72	75	78	108	114	119	125	130
0·50	116	120	125	130	135	81	85	88	91	94	135	141	146	152	157
0·60	139	144	149	153	158	98	101	104	107	111	162	168	173	179	184
0·70	162	167	172	176	181	114	117	120	124	127	190	195	200	206	211
0·80	186	190	195	200	204	130	133	137	140	143	217	222	227	233	238
0·90	209	214	218	223	227	146	150	153	156	159	244	249	255	260	265
	1 cc. = 232·1					1 cc. = 162·5					1 cc. = 270·8				

The clinical significance of estimation of acetone bodies in blood.

At the present date when doubt has been thrown on the view that the ketone bodies are the most important factor in the causation of diabetic coma, a simple method of estimating the degree of ketonaemia is likely to be useful both for the investigation of the aetiology of coma and for the treatment of individual cases. It is well known that when the diminution of alkali reserve does not run parallel with the concentration of ketone bodies in blood, the clinical condition (severity of coma *etc.*) accords with the former rather than with the latter and in such cases beneficial effects have been obtained by

administering alkalis. It would be worth while therefore to estimate the degree of ketonaemia in order to decide whether insulin should be pushed further or a trial given to alkalis. The degree of ketonuria may be of no help since it is now definitely known that if the kidneys have been damaged they may fail to excrete ketone bodies.

In view of the finding of Starr and Fitz [1924] that in several cases of severe diabetes investigated by them there was evidence of the presence of some organic acid other than the known keto-acids, it would be interesting to calculate the sum of the alkali reserve as determined and the diminution of CO_2-combining power of blood attributable to the fixation of base by the keto-acids. One molecule of a keto-acid may be taken to displace one molecule of CO_2 from one molecule of $NaHCO_3$ according to the formula

$$HA + NaHCO_3 \rightarrow NaA + H_2O + CO_2.$$

Thus keto-acids corresponding to 58·05 mg. acetone would displace 44 mg. CO_2, *i.e.* 22·4 cc. at N.T.P. Therefore the concentration (as mg. acetone per 100 cc.) of acetone bodies in blood multiplied by $\frac{22{\cdot}4}{58{\cdot}05}$, *i.e.* 0·385, will give the diminution of CO_2-combining power due to the presence of the keto-acids. When this is added to the alkali reserve as determined, a normal figure should be obtained if the acidosis is principally caused by acetone bodies. Conversely if a figure much lower than normal is obtained, there is presumptive evidence of the presence of other organic acids in the blood. In such cases perhaps too much reliance on insulin would not be justified and administration of alkalis might be of help. (The reasoning employed here is essentially the same as that of Starr and Fitz, only expressed in a different way.)

During recovery from coma acetone bodies in blood fluctuate very widely. It is possible that the sudden great rises met with, while the clinical condition is steadily improving, are due to the administration of glucose. On several occasions, while studying blood-sugar curves in diabetics, the writer has observed a marked rise in ketonaemia and ketonuria along with the rise of blood-sugar. (The ketone bodies fall however more rapidly than blood-sugar.)

The writer acknowledges his gratitude to Dr George Graham and Dr G. A. Harrison of St Bartholomew's Hospital, London, and to the authorities of the Haffkine Institute and King Edward Memorial Hospital, Bombay, for giving him facilities to carry out this investigation.

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CLXVI. EFFECT OF ADDITION OF SALTS ON THE ISOELECTRIC POINT OF PROTEINS. III.

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(*Received July 8th, 1932.*)

In the present paper, experiments are described on the influence of electrolytes on the critical concentration, C_K, of alcohol necessary to flocculate gelatin solutions, and on the viscosity of aqueous and aqueous-alcoholic gelatin solutions.

Experimental.

Purification of gelatin. The gelatin taken, purchased from Coignet Père et Fils, had 1·49 % of ash which consisted almost exclusively of calcium phosphate, with traces of iron. After purification, the ash content fell to 1·1 %; for the concentrations of gelatin used in our experiments, *viz.* 0·15 and 0·9 %, this would correspond with 0·0165 and 0·099 g. per litre of solution, *i.e.* the concentrations of $Ca_3(PO_4)_2$ originally present were 0·00032 and 0·00192 *N*.

A filtered 4 % solution of gelatin in warm water was poured in a thin stream into 4 volumes of alcohol. The flocculent precipitate was left in contact with alcohol for 24 hours, when the solution was violently agitated, and allowed to settle. The supernatant clear solution was then decanted, and the precipitate was shaken with fresh alcohol and filtered. The residue after evaporation of alcohol was redissolved and the gelatin was reprecipitated as before, with the difference that the duration of contact with alcohol before shaking was increased to 48 hours. The residue after filtration was washed 4 times with 96 % alcohol, allowing the suspension to settle 3 hours before every decantation. The product so obtained was spread in a thin layer on sheets of glass, and dried *in vacuo* over calcium chloride, giving a white impalpable powder, the properties of which remained unchanged by further treatment with alcohol.

The action of electrolytes on gelatin solutions was examined by adding various concentrations of salts to the clear solution, and then adding sufficient 96 % alcohol to produce a milky opalescence. The volume of alcohol necessary (in future designated by *V*) is fairly constant for a given concentration of gelatin and electrolyte, and sufficiently reproducible results can, with a little experience, be obtained without any difficulty. The procedure adopted was as follows: 2 cc. of electrolyte solution were added to a series of 70 cc. test-tubes (pyrex) each of which contained 3 cc. of 0·5 % gelatin; the volume was made

up to 10 cc., and alcohol was added from a burette, in small portions, shaking after the addition of every portion.

Viscosity was determined using Ostwald's viscosimeter, and calculating the results from the simplified formula $\eta = T'/T$, where T' and T are the rates of flow of the gelatin solution examined, and of a solution of electrolyte of the same concentration as in the gelatin solution. Determinations were made at 40°, in view of Davis and Oakes's [1922] observation that gelation, with consequent augmentation of viscosity, does not take place at temperatures higher than 38·1°. 3 cc. of electrolyte solution were added to 27 cc. of 1 % gelatin buffered to the appropriate p_H or of a mixture of equal volumes of 2 % gelatin and 65 % alcohol in 50 cc. flasks, which were then stoppered, and allowed to stand for 1 hour at 40° before viscosity was measured. Determinations were made in a glass-walled thermostat at 40° ± 0·1°, and the values given are the mean of 4–6 determinations.

1. *Influence of* p_H *and salts on* V.

The results obtained are illustrated by the curve shown in Fig. 1. At the isoelectric point (p_H 4·85), V is at a minimum, *viz.* 6 cc. (30 %), whilst at

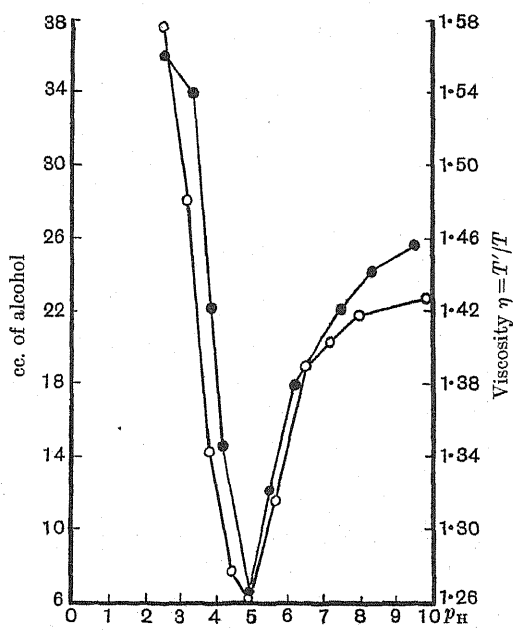


Fig. 1.

○——○ Turbidity curve. ●——● Viscosity curve.

p_H 4·3, 14·5 cc. (56 %); at p_H 3·8, 22 cc. (66 %); at p_H 3·4, 34 cc. (74 %); and at p_H 2·6, 37 cc. (75·5 %) are necessary to produce opalescence. Similarly, at p_H above 4·85, V rises from 12 cc. at p_H 5·5 to 18 cc at p_H 6·3, 22 cc. at p_H 7·5, 24 cc. at p_H 8·4 and 25·5 cc. at p_H 9·5.

Table I. *Action of $MgCl_2$.*

Conc. of $MgCl_2$ N	Least volume of alcohol required to produce turbidity at p_H						
	3·87	4·49	4·84	6·15	7·63	8·97	9·63
0	22	10	6·5	18·5	22	24	25·5
0·002	—	—	7·5	13	14·5	15·5	15·5
0·004	—	—	8·5	11·5	13	13·5	15·5
0·006	—	—	10	11	12·5	12·5	13·5
0·008	—	—	11·5	11·5	12	12	12·5
0·010	—	—	13	12	12	12	12
0·02	23	15	14·5	12·5	12·5	12	12·5
0·04	24	18	16	14·5	14·5	14	14
0·06	24·5	19	18	15	15·5	15·5	15
0·08	25	20	19·5	16·5	16	16·5	16·5
0·10	25	21	20	17	17	17·5	17

The action of $MgCl_2$ is different according to its concentration and to the p_H of the medium. Thus at and below the isoelectric point (I.P.) $MgCl_2$ increases V to an extent diminishing with diminishing p_H, V rising from 6·5 to 20 cc. at p_H 4·85, from 10 to 21 cc. at p_H 4·49 and from 22 to 25 cc. at p_H 3·87. Above the I.P., V is smaller in the presence of $MgCl_2$ than in its absence; for every p_H, however, a minimum V is obtained for a certain concentration of $MgCl_2$, above and below which V is greater and may, at p_H close to the I.P., even exceed the value found in the absence of electrolytes. The smallest value of V observed at p_H 6·15 is 11 cc. in the presence of 0·006–0·01 N $MgCl_2$, as compared with the value of 6 cc. found in the absence of salts at p_H 4·8, showing that the charge on the particles cannot be reduced by addition of $MgCl_2$ to that found at the isoelectric point.

Table II. *Action of Na_2SO_4.*

Conc. of Na_2SO_4 N	Least volume of alcohol required to produce turbidity at p_H						
	3·20	4·43	4·73	5·4	6·1	6·7	7·1
0	35	14·5	6	13	18·5	20	21·5
0·002	17	10	19·5	23	31·5	34	36·5
0·004	15	9·5	22	25	33	36·5	38
0·006	13	8·0	23	25	33	36	37
0·008	12·5	8·5	24	25·5	31·5	34	36
0·01	12	9·0	24	25·5	30	33	33·5

0·006 N Na_2SO_4 increases V from 6 to 24 cc. at the I.P., and decreases it below the I.P. from 35 to 12 cc., at p_H 3·2. Above the I.P., Na_2SO_4 greatly increases V, the values of which are considerably higher at all values of $p_H > 4·85$ than in the absence of electrolytes. It is not possible on the basis of the experimental material available to decide whether this effect is due to formation of $CaSO_4$ by interaction between Na_2SO_4 and Ca combined with gelatin, or to augmentation of the charge on the colloidal particles. As the concentration of Na_2SO_4 is increased, V rises to a maximum value, and then falls; thus, at p_H 7·1 V rises from 21·5 cc. in the absence of Na_2SO_4 to 38 cc. in the presence of 0·004 N Na_2SO_4, and falls to 33·5 cc. for 0·01 N Na_2SO_4.

The action of $CaCl_2$ was studied only at p_H 5·6 and was found to be analogous to that of $MgCl_2$. Thus V falls from 14 to 7 cc. when $CaCl_2$ is added to 0·003 N, rising to 19·5 cc. when the concentration is increased to 0·1 N.

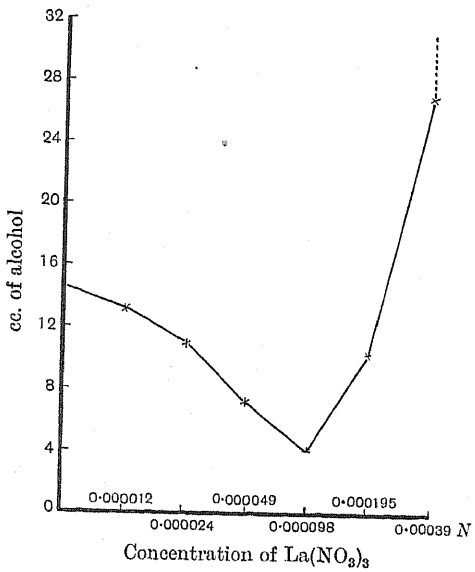


Fig. 2. Influence of $La(NO_3)_3$ on the volume of alcohol required to produce turbidity of gelatin solutions at p_H 5·5.

In the case of $La(NO_3)_3$ (studied only at p_H 5·5), V falls sharply from 14 to 3·5 cc. as the concentration of $La(NO_3)_3$ is increased from 0 to 0·0001 N, and again rises sharply to 26 cc. in 0·0004 N $La(NO_3)_3$. KCl, NaCl and $MgSO_4$ have only a very slight effect on the value of V (Table III).

Table III. *Action of KCl, NaCl and $MgSO_4$ at* p_H *5·3.*

Conc. of salt N	Least volume of alcohol required to produce turbidity in the presence of		
	KCl	NaCl	$MgSO_4$
0	14	14	14
0·00075	14	14	14
0·0015	14	14	14
0·0031	14	14	14
0·0062	14	14	13·5
0·0125	14	14	13·5
0·025	15	14	13·6
0·05	16	15	13·0
0·1	17	16	13·0

A comparison of the actions of $MgCl_2$, Na_2SO_4 and $MgSO_4$ on portions of the same gelatin solution at p_H 5·6 showed (Fig. 3) that as the concentrations of salts giving rise to bivalent anions and univalent cations are increased,

V at first increases and then diminishes; in the case of those giving univalent anions and bivalent cations, V at first falls and then rises, whilst bi-bivalent electrolytes have practically no action.

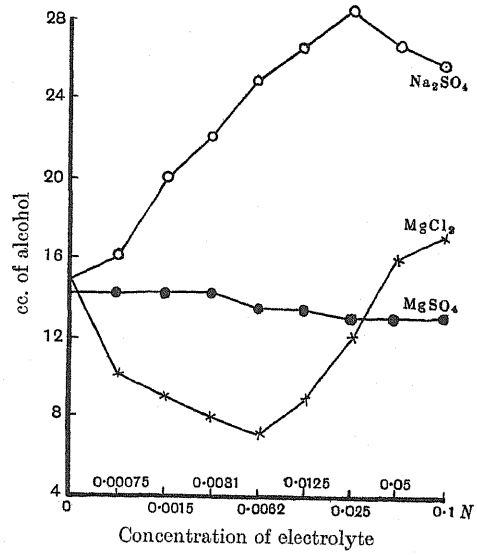


Fig. 3. No. of cc. of alcohol necessary to produce turbidity in the presence of Na_2SO_4, $MgCl_2$ and $MgSO_4$.

2. *Influence of electrolytes on viscosity.*

The viscosity-p_H curve has a minimum at the I.P., η being 1·575 at p_H 2·5, 1·480 at p_H 3·3, 1·340 at p_H 3·8, 1·275 at p_H 4·5, 1·260 at p_H 4·84, 1·315 at p_H 5·7, 1·388 at p_H 6·5, 1·400 at p_H 7·3, 1·416 at p_H 8·0 and 1·424 at p_H 9·8 (Fig. 1).

Table IV. *Influence of electrolytes on viscosity.*

Conc. of salt N	Viscosity at p_H 4·0 in the presence of			Viscosity at p_H 6·4 in the presence of		
	NaCl	KCl	LiCl	NaCl	KCl	LiCl
0	1·370	1·370	1·370	1·367	1·367	1·367
0·0006	1·368	1·370	1·370	1·360	1·352	1·307
0·0012	1·355	1·366	1·364	1·352	1·325	1·310
0·0025	1·337	1·345	1·345	1·337	1·310	1·313
0·005	1·327	1·329	1·329	1·323	1·305	1·308
0·01	1·313	1·313	1·310	1·311	1·308	1·311
0·02	1·292	1·292	1·292	1·308	1·316	1·308
0·04	1·283	1·290	1·291	1·314	1·320	1·314

At p_H below the I.P. the action of uni-univalent electrolytes is of the same type for the three salts studied (KCl, NaCl, LiCl); these salts all lower η to about the same extent, and the same applies to $MgCl_2$, whilst the action of $La(NO_3)_3$ is smaller than is that of alkali or alkaline earth salts. $K_3Fe(CN)_6$

Table V. *Influence of KCl, $MgCl_2$, $La(NO_3)_3$, Na_2SO_4 and $K_3Fe(CN)_6$ on viscosity at p_H 3·4.*

Conc. of salt (N)	KCl	$MgCl_2$	$La(NO_3)_3$	Na_2SO_4	$K_3Fe(CN)_6$
0	1·324	—	—	—	—
0·0006	1·322	1·316	1·323	1·310	1·252
0·0012	1·314	1·306	1·313	1·308	1·186
0·0025	1·292	1·288	1·303	1·270	1·150
0·005	1·270	1·264	1·292	1·227	} Partial
0·01	1·262	1·254	1·283	1·216	} coagulation
0·02	1·252	1·246	1·270	1·210	1·131
0·04	1·254	1·246	1·257	1·210	1·140

Table VI. *Influence of KCl, $MgCl_2$, $La(NO_3)_3$, Na_2SO_4 and $K_3Fe(CN)_6$ on viscosity at $p_H >$ isoelectric point.*

Conc. of salt N	p_H 6·5			p_H 7·4		p_H 8·5
	KCl	$MgCl_2$	$K_3Fe(CN)_6$	$MgCl_2$	Na_2SO_4	$La(NO_3)_3$
0	1·378	1·378	1·378	1·400	1·400	1·420
0·0006	1·373	1·357	1·367	1·384	1·094	1·408
0·0012	1·355	1·334	1·348	1·360	1·090	1·366
0·0025	1·334	1·322	1·342	1·334	1·378	1·302
0·05	1·314	1·303	1·340	1·284	1·356	1·246
0·01	1·306	1·289	1·340	1·240	1·334	1·236
0·02	1·308	1·279	1·336	1·218	1·318	1·244
0·04	1·310	1·276	1·338	1·218	1·310	1·254

Table VII. *Influence of salts on viscosity in aqueous-alcoholic systems.*

Conc. of salt N	p_H 3·8		p_H 4	p_H 4·5			p_H 6·01	
	Na_2SO_4	$K_3Fe(CN)_6$	$La(NO_3)_3$	KCl	$MgCl_2$	$K_3Fe(CN)_6$	Na_2SO_4	$MgCl_2$
0	2·794	2·794	2·753	2·698	—	—	2·623	2·623
0·0003	2·742	2·671	—	—	—	—	2·616	2·580
0·0006	—	—	2·770	2·686	2·694	2·650	—	—
0·0007	2·686	2·600	—	—	—	—	2·613	2·531
0·0012	—	—	2·792	2·672	2·686	2·564	—	—
0·0015	2·638	2·522	—	—	—	—	2·606	2·516
0·0025	—	—	2·800	2·664	2·660	2·476	—	—
0·003	2·620	Turbidity	—	—	—	—	2·611	2·508
0·005	—	—	2·815	2·650	2·640	Turbidity	—	—
0·006	2·618	2·514	—	—	—	—	2·613	2·504
0·01	—	—	2·822	2·642	2·632	—	—	—
0·0125	2·622	2·518	—	—	—	—	2·617	2·504
0·02	—	—	2·823	2·640	2·626	2·436	—	—
0·025	2·628	2·526	—	—	—	—	2·626	2·509
0·04	—	—	2·826	2·640	2·622	2·444	—	—

Conc. of salt N	p_H 6·5		p_H 7·2	p_H 7·5		
	Na_2SO_4	$K_3Fe(CN)_6$	$La(NO_3)_3$	KCl	$MgCl_2$	$K_3Fe(CN)_6$
0	2·698	2·698	2·730	—	2·750	—
0·0003	2·698	2·705	—	—	—	—
0·0006	—	—	2·580	2·746	2·720	2·754
0·0007	2·690	2·706	—	—	—	—
0·0012	—	—	2·514	2·742	2·670	2·754
0·0015	2·692	2·708	—	—	—	—
0·0025	—	—	2·468	2·732	2·630	2·758
0·003	2·693	2·712	—	—	—	—
0·005	—	—	Turbidity	2·724	2·610	2·760
0·006	2·697	2·711	—	—	—	—
0·01	—	—	Turbidity	2·718	2·594	2·758
0·0125	2·699	2·713	—	—	—	—
0·02	—	—	2·474	2·718	2·598	2·760
0·025	2·701	2·719	—	—	—	—
0·04	—	—	2·486	2·710	2·602	2·758

lowers η more than do the previously mentioned electrolytes; this salt, however, produces partial flocculation at concentrations of 0·005 and 0·01 N.

Above the I.P., salts possessing univalent cations and multivalent anions (Na_2SO_4, $K_3Fe(CN)_6$) have a feebler action than have uni-univalent salts (KCl), which, again, are less active than are salts of multivalent metals ($La(NO_3)_3$). In the case of $La(NO_3)_3$ η falls with concentration to a minimum at 0·01 N, again rising at higher concentrations of salt (Table VI).

In aqueous-alcoholic media uni-univalent electrolytes lower η on both sides of the I.P. Salts giving rise to multivalent ions of opposite charge from the protein have a more marked action, which augments with increase in the valency. Thus, below the I.P., Na_2SO_4 has a more powerful action than has KCl, whilst that of $K_3Fe(CN)_6$ is, up to a certain concentration, still more marked, partial flocculation taking place. At higher concentrations of $K_3Fe(CN)_6$ its flocculating action ceases, and η again rises slightly. $La(NO_3)_3$ causes increase in η below the I.P. Above the I.P. depression of η increases with the valency of the cation of multi-univalent salts (KCl, $MgCl_2$, $La(NO_3)_3$), Na_2SO_4 is practically without action, and $K_3Fe(CN)_6$ very slightly increases η.

Summary and discussion.

1. The previous finding that the concentration (C_K) of alcohol necessary for the flocculation of gelatin sols is smallest at the isoelectric point (p_H 4·8) has been confirmed for purified preparations of gelatin. The flocculation-alcohol concentration curve (Fig. 1) has a greater slope at $p_H < 4{\cdot}8$ than at $p_H > 4{\cdot}8$. Negatively charged micelles are more readily coagulated by alcohol than are positively charged ones.

2. The value of C_K at the isoelectric point is increased in the presence of $MgCl_2$ or Na_2SO_4, whilst $MgSO_4$ is without effect. The action of uni-multivalent salts at the isoelectric point can, according to Pauli [1930], be ascribed to combination between the salt and zwitterions, yielding products of the type $XH_3N.R.CO_2M$, with the production of double salts of different activities, to which the charge of the particles is due.

3. At all p_H values salts giving rise to ions of the same valency do not affect the value of C_K.

4. At $p_H < 4{\cdot}8$ salts yielding multivalent anions and univalent cations lower C_K at small concentrations, and slightly raise it at $p_H > 4{\cdot}8$. This effect is due to the charge of the colloidal particles; the greater the original charge the greater the percentage lowering of C_K. Thus V, the volume of alcohol necessary to produce turbidity, is 14 cc. at p_H 4·25, whilst in the presence of 0·06 N Na_2SO_4 V is 8 cc., and at p_H 3·2 V falls from 35 to 13 cc.

Salts yielding univalent anti-ions and multivalent iso-ions, *e.g.* $MgCl_2$ at p_H 4·8, considerably raise the value of C_K to an extent which is greater the closer the p_H is to 4·8. This effect may be ascribed to increase in the free charge of gelatin particles. The opinion that this phenomenon is analogous to

that of the formation of double salts of diminished activity, as with protein (*e.g.* $NH_3Cl.R.CO_2\frac{1}{2}Mg$), with consequent diminution in the negative charge, lacks experimental proof, although it is in agreement with the zwitterion theory, and with Kruyt's [1929] observation of augmented cataphoresis of gelatin in the presence of Na_2SO_4 at p_H 4·4.

5. The action of salts at $p_H > 4{\cdot}8$ is analogous to that at $p_H < 4{\cdot}8$, with the difference that anions are anti-ions, and cations iso-ions. Augmentation of C_K by Na_2SO_4 can be observed at p_H as high as 7·1. The further the p_H is from 4·8, the more marked is the action of univalent anti-ions. A certain concentration of salt can be found giving maximum increase in C_K; the value of this optimum concentration diminishes with increasing p_H.

6. The dual action of a salt is the more marked the greater is the difference between the valencies of its ions. Thus $La(NO_3)_3$ at p_H 5·3 in low concentrations lowers C_K, and hence the charge of the particles, and at higher concentrations augments it to values even higher than are found in the absence of $La(NO_3)_3$. According to the interpretation of Przyłęcki [1931] and of Przyłęcki and Giedroyć [1931] this effect is due to supercharging. Since the discharging of the colloidal particles takes place at very low concentrations of salt (0·0004 %), the supercharging of the particles by the anti-ions similarly takes place at low concentrations of salt, with consequent absence of discharging action by higher concentrations. The action of $CaCl_2$ is similar to that of $La(NO_3)_3$ but much feebler, supercharging being observed at a concentration of 0·006 *N*. Were this phenomenon to be ascribed to reduction in the charge due to ionised carboxylic groups, it would have, according to the zwitterion theory, to be assumed that only positively charged $—NH_3^+$ groups remain, to which the charge of the particles is due.

The reason for the increase in C_K at p_H 7·1 in the presence of Na_2SO_4 is not obvious. This effect may be due to the action of SO_4 on the remaining dissociated $—NH_3^+$ groups, or to the reaction:

$$\text{Ca gelatinate} + Na_2SO_4 \rightleftharpoons Na_2 \text{ gelatinate} + CaSO_4.$$

7. The p_H-viscosity curves of aqueous systems are very similar to the p_H-V curves, minimum viscosity being at p_H 4·85, with a rapid rise in viscosity on the acid side of this point, and a gentle rise on the alkaline side (Fig. 1). This similarity is due to the identity of the factors leading to change in the charge of the particles, both increase in C_K and in viscosity being ascribable to increase in the charge.

8. The action of salts on the viscosity of gelatin sols is not in agreement with their effect on the value of C_K. Salts yielding univalent iso-ions and anti-ions diminish viscosity at all values of p_H. The actions of salts yielding the same anti-ions and different iso-ions are very nearly the same irrespective of the salt taken (KCl, LiCl, NaCl at p_H 4·0), whilst where the anti-ions are different but the iso-ion identical (KCl, LiCl, NaCl at p_H 6·4), the magnitude of the effect obtained varies with the different salts.

9. Salts yielding multivalent anti-ions and univalent iso-ions depress viscosity to an extent increasing with the valency of the anti-ion. As the concentration of salts of this type is increased, viscosity falls to a minimum value, again rising with higher concentrations.

10. Salts yielding multivalent iso-ions and univalent anti-ions ($K_3Fe(CN)_6$) at p_H 6·5 diminish viscosity to a smaller extent than do uni-univalent salts (KCl).

11. The action of salts on the viscosity of aqueous-alcoholic solutions of gelatin is different from that in the absence of alcohol. Under certain conditions viscosity is increased by addition of salts. The Schulze-Hardy rule is followed in such systems, although in certain cases the viscosity at first falls and then rises as the concentration of salt is increased. Salts yielding univalent anti-ions and multivalent iso-ions increase viscosity at all concentrations ($K_3Fe(CN)_6$ and Na_2SO_4 at $p_H > 4{\cdot}8$, and $La(NO_3)_3$ at $p_H < 4{\cdot}8$).

12. No explanation of the difference in the action of salts on viscosity in aqueous and in aqueous-alcoholic media is, in the present state of knowledge of this subject, apparent.

The author has pleasure in acknowledging his indebtedness to Prof. St J. Przyłęcki, at whose suggestion this research was undertaken, and under whose direction it was performed.

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CLXVII. STUDIES ON PLANT AMYLASES.
I. THE EFFECT OF STARCH CONCENTRATION UPON THE VELOCITY OF HYDROLYSIS BY THE AMYLASE OF GERMINATED BARLEY.

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It is clear that, for the determination of enzymic reaction-velocity under readily definable conditions, the states near the initiation of the reaction are decidedly the best.

Usually however this part of the progress has been left unexplored and delineation of it has been based merely upon empirical expressions of doubtful value. In the present paper data have been directly collected within a few minutes of the origination of the reaction on the mixing of enzyme and substrate. From these data "initial slopes" are determined graphically and used as a measure of reaction-velocity. In experiments of this short duration the difference between apparent and true zero-time becomes significant.

The starch-amylase system. Description of an "initial slope" method.

Of the various criteria which have been used for following the progress of starch degradation in the presence of amylase, the production of reducing sugar can be most accurately measured. When there is a possibility, as in this reaction, that the final product is the result of a series of transformations, it would be preferable to derive reaction velocity values from data on the disappearance of the original substrate, rather than the accumulation of the final product. In the absence of methods, however, for the accurate determination of the unchanged starch, it is necessary to rely upon determinations of the reducing sugar produced for precise information concerning the progress of the reaction. The objections to this procedure are minimised if the reaction velocity determinations are restricted to the initial period before there is an appreciable falling-off in rate, since it can then be concluded that one particular stage in a possible series of reactions is being measured.

[1] This work was carried out during the tenure of a Studentship from the Royal Commissioners of the Exhibition of 1851, to whom the author offers grateful acknowledgment.

An analysis of a large number of published experimental data on amylase action has led to the conclusion that many methods which have been used for obtaining reaction velocity values frequently fail to give a reliable measure of the initial reaction velocity, and that the results are accordingly of limited significance. An "initial slope" method has therefore been used in an attempt to obtain more reliable data on the action of certain plant amylases.

The success of such a method depends upon (1) the use of a suitable analytical procedure for the precise determination of small amounts of reducing sugar, and (2) a careful standardisation of the technique for mixing enzyme and substrate, withdrawing samples, and arresting the reaction in these samples.

(*a*) *Sugar determination.*

A trial of various existing micro-methods of sugar analysis indicated the desirability of developing an analytical procedure adapted to the specific requirements of the present problem, since existing methods were found to be inadequate either because the range was too limited or the determination of the smaller amounts of sugar was unreliable. Accordingly a method was developed based on that of Hagedorn and Jensen. In this modified procedure, which has been described earlier [Hanes, 1929], the range of the original method was increased about tenfold. Its precision and range have proven satisfactory for the present purpose. It has been found, moreover, that the alkaline ferricyanide reagent used in this method (solution A) is effective for arresting the action of amylase on starch.

The sugar which is formed from starch after prolonged action of the barley amylase preparations used is almost exclusively maltose. It has been assumed, although not experimentally demonstrated, that maltose is also the sugar which appears in the early stages of the reaction. The sugar method was therefore standardised for maltose, and the reducing power is expressed in terms of maltose.

(*b*) *Standardised procedure for following the early part of the reaction.*

The reaction is carried out in 75 cc. Erlenmeyer flasks, supported in a water-thermostat. Ordinarily the temperature is maintained at 25°, although it has been advantageous for certain purposes to use lower temperatures.

A total digest volume of 29 cc. has been found suitable for most of the work. This is composed of 25 cc. of substrate preparation, 2 cc. of buffer solution, and 2 cc. of enzyme solution. This volume allows 5 samples to be withdrawn for maltose determination.

The solutions used, and the digests themselves, are kept free from bacterial contamination by saturation with toluene, and the pipettes are guarded with thick plugs of cotton wool, to exclude traces of saliva.

Before starting a digest, the solutions to be used and the reaction flasks are allowed to come to the temperature of the water-bath, and 5 cc. portions of the ferricyanide solution A, for sugar analysis, are pipetted into a series

of numbered boiling-tubes. A digest is prepared by pipetting 25 cc. of substrate and 2 cc. of buffer solution into the reaction flask. Finally the enzyme is run in from a 2 cc. pipette. At the moment of removing the finger to start the delivery of the pipette, a stop-watch is started. This is referred to as "zero time." The delivery of the pipette takes about 8 seconds; when it is nearly empty the last drops are blown out, the tip being held against the wall of the flask. It is then tapped three times against dry portions of the wall. The flask is then removed from the rack and shaken vigorously to wash down the walls and mix the contents. This is usually completed about 20 seconds after zero time.

A clean dry 5 cc. pipette is now filled with digest liquid and allowed to drain back into the flask. It is then refilled, and the level adjusted to the mark. The tip is then freed from adhering drops of liquid and inserted into one of the boiling-tubes, touching the wall about 2 cm. above the level of solution A. At a convenient point on the stop-watch, usually at an even tenth-second, the delivery of the sample is started into the tube. The delivery occupies about 7 seconds, after which the pipette is allowed to drain a further 10 seconds, the tip still in contact with the wall. It is then tapped five times against dry portions of the wall, and removed. The boiling-tube is now inclined in the hand and rotated to wash down the wall. The time of the beginning of the delivery, and the number of the tube are noted. The procedure for subsequent samples is identical. The same pipette is used throughout each digest without washing with water, but before taking each sample it is rinsed out with the digest liquid.

Ordinarily the tubes are left for less than 15 minutes before continuing with the procedure for maltose estimation, although no appreciable differences are found if they are left longer.

When facility with the technique had been acquired, the delivery of the first sample into the "killing" solution could be started as early as 0·6 minute after zero time, all operations being carried out in the standard way. For most purposes, it has been found satisfactory to take the first sample at about 1·0 minute after zero time, and the second and third at about 3·0 and 6·0 minutes respectively. However, it is necessary to bear in mind, when deciding upon the timing of digest samples, that the aim of the procedure is to establish the initial slope of the progress curve. Consequently, if for any reason it is expected that the reaction velocity of a particular digest will fall off relatively earlier, then samples are taken at shorter intervals. An example of this procedure is to be seen in Digest No. 6 (p. 1413) in which the times of successive samples were 0·7, 1·9, 3·1 and 4·3 minutes after zero time. The ideal timing of samples presupposes a knowledge of the particular reaction system under observation.

(*c*) *The derivation of reaction velocity values from the data.*

It is found on plotting the data obtained by this procedure, that the progress of sugar formation is seldom strictly rectilinear even during the brief

initial periods under observation, although under certain conditions the falling-off in velocity may be slight. Usually a progressive decline in reaction velocity is apparent from the beginning, and it is evident that the earliest measured slope (that is, the slope during the interval between the first and second samples) must be lower than the ideal initial slope. It is accordingly desirable to attempt to obtain by extrapolation a slope value which will approximate more closely to the ideal initial slope.

The extrapolation is carried out by plotting the progress-time data on a large scale graph, and joining the points by a carefully-drawn curve. A black silk thread is stretched across the paper and its position and direction adjusted by sighting along the curve and the thread so that it passes through the earliest experimental point, and a portion of the thread forms an extrapolation of the curve back to zero time. The slope of the thread can then be accurately determined by readings on the squared paper. Three such determinations are usually made and the average value is taken as a measure of the initial reaction velocity. This procedure gives a slope value at a point approximately half-way between zero time and the time of the first sample, in practice, about 20 seconds after zero time.

In explaining the reason for adopting this particular method of extrapolation it is necessary to mention certain complications which arise as a result of the extreme shortening of the time scale which is effected in the present procedure. Two lag effects, which are negligible when the time intervals of sampling are longer, may now become appreciable; these effects are due to (1) the "initial lag" phase after zero time, representing the time for the delivery of the enzyme and its mixing with the substrate and possibly, in addition, for the attainment of equilibrium between enzyme and substrate; and (2) the "killing" lag, representing the time required for arresting the reaction in each digest sample after starting the delivery into the alkaline ferricyanide solution. These lag phenomena have the effect of causing displacements of empirical progress curves in time, the initial lag producing a displacement to the right (when the zero point of the time axis is at the left), the "killing" lag, a displacement to the left. In extrapolating for initial slope values, an analysis has shown that the aim should be to measure the slope at the intersection between the extrapolated progress curve and the horizontal line representing the level of the initial reducing power of the digest components. Consequently, unless the two opposing displacements in time are equal, a "corrected origin" is required for extrapolation. For the present data, the "corrected origin" probably occurs about 10–20 seconds after zero time. The method of extrapolation described above gives a measure of the slope at about 20 seconds after zero time and this has been adopted as a working approximation to the theoretical ideal.

THE EFFECT OF STARCH CONCENTRATION UPON THE REACTION VELOCITY.

The relationship between substrate concentration and reaction velocity in an enzyme-substrate system is of considerable theoretical importance. Briggs and Haldane [1925], in extending the earlier formulations of the Michaelis theory of enzyme action [Michaelis and Menten, 1913], consider the reaction $A \rightarrow B$, unimolecular as regards A, catalysed by an enzyme E. They suppose one molecule of A to combine reversibly with one molecule of E forming an enzyme-substrate compound, which then decomposes irreversibly into free enzyme and B, the product of the reaction. The system considered may be represented:

$$A + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AE \xrightarrow{k_3} E + B,$$

the specific constants for the different reactions being as indicated, k_1, k_2 and k_3.

By applying the law of mass action, an expression is deduced relating reaction velocity and substrate concentration, which would be valid so long as the molar concentration of enzyme is negligibly small compared with that of substrate. This expression is:

$$v = V_\infty a/(a + K_s),$$

where a is the molar substrate concentration,

v, the reaction velocity,

V_∞, the maximum velocity of the system which would be attained when the substrate concentration is so large that the whole of the enzyme exists in the form AE[1],

and K_s is the ratio $(k_2 + k_3)/k_1$.

The relationship between reaction velocity and substrate concentration predicted from this hypothesis is a rectangular hyperbola.

Two attempts have been made to test the applicability of this hypothesis to the action of barley amylase. Sjöberg and Erikson [1924], using as substrate preparations of "amylose" and "amylopectin" prepared according to Gatin-Gruzewska, came to the conclusion that the relationship is of the form predicted by the theory. The experimental evidence offered in support of this conclusion is very limited and far from satisfactory on other grounds. Eadie [1926] in re-investigating the question, using Lintner's soluble starch as substrate, came to the conclusion that the relationship is of an entirely different kind from that predicted by the theory, since he observed that with certain low starch concentrations no reaction occurred. He was therefore of the opinion that the Michaelis theory was not applicable to the action of barley amylase. The reason for the anomalous nature of Eadie's results will be made clear later.

[1] This assumes that no secondary effects of high substrate concentration arise, such as water shortage.

Experimental.

A study of the effects of varying the substrate concentration has been carried out, using the initial slope method as detailed on p. 1407. Several different preparations of amylase from germinated barley have been used.

Method of preparing amylase from barley.

Barley of known variety, locality and season of growth has generally been used. A weighed sample of selected grains, usually 100 or 200 g., is washed thoroughly and allowed to soak in distilled water for about 10 hours. It is then spread in a thin layer on moist filter-paper and set away to germinate in a dark moist chamber. The grains are occasionally stirred and moistened during the germination period. When the plumules, still within the seed coat, have grown to about 1/2 to 3/4 of the length of the seed, the grains are placed in a beaker and killed by stirring with a little toluene, and then ground to a paste in a fine mincer.

This material is then extracted in an ice-chest with dilute acetic acid (usually 0·1 %) for a period varying from 6 to 24 hours, with occasional stirring. The coarser sediment is then removed either by squeezing through fine cloth in a press, or by centrifuging. The turbid liquid is then filtered until almost clear. The enzyme is now precipitated by adding sufficient 95 % ethyl alcohol to make a final concentration of 70 % by volume. The liquid is set away in an ice-chest in a tall vessel for 12–24 hours, during which time the precipitate settles. The bulk of the clear supernatant liquid is then decanted. The remainder is filtered through Whatman's No. 50 paper on a Büchner funnel. The final pinkish residue is allowed to drain on the filter for 15 minutes. The paper bearing the residue is then removed and ground up in a mortar with distilled water. The liquid is poured off and filtered. The successive residues (and papers) are repeatedly extracted to remove the water-soluble fraction. The clear, faintly yellow filtrate is then made up to a definite volume, and toluene is added as a preservative. The preparations obtained in this way have a very low content of reducing sugar. They remain clear for several days but usually on prolonged storage a small flocculent precipitate appears which is inactive. The hydrogen ion concentration is generally in the range p_H 6·4–6·8 and the buffering power is slight. In different preparations the total dry weight has represented from 0·1 to 0·35 % of the dry weight of the original barley.

On occasion, further purification has been attempted by dialysis, reprecipitation, fractional precipitation, and by adsorption on kaolin. Reference to these preparations will be reserved for a later paper.

Exp. 1. Enzyme. Amylase 1, prepared from germinated barley. (See Appendix 1.)

Substrate. Two solutions of soluble starch, 1·25 and 0·145 %, were used in this experiment. They were freshly prepared from a sample of Lintner's soluble starch (Merck) which had been dried to constant weight at room

temperature over sulphuric acid. An amount of starch, sufficient to make 1 litre of the required concentration, is suspended in a small volume of water and then washed into 600 cc. of boiling water. The whole is then brought to the boil, boiled for 8 minutes, cooled and made up to 1 litre. Toluene is added.

Buffer. 2 cc. of acetic acid-sodium acetate buffer, $M/5$ in acetate, in each digest of 29 cc., making the final concentration $M/73$ approximately. The final p_H was 4·71–4·73 in all digests[1]. The reaction temperature was 25·0°.

Digests were set up as shown in Table I, and the data obtained from them are given in Table II.

Table I.

Digest No.	1	2	3	4	5	6	7	8	9	10	11	12
1·25 % soluble starch cc.	25	15	10	5	3	2	—	—	—	—	—	—
0·145 % soluble starch cc.	—	—	—	—	—	—	10	8	6	3	1	1
Water cc.	—	10	15	20	22	23	15	17	19	22	24	24
Buffer solution cc.	2	2	2	2	2	2	2	2	2	2	2	2
Amylase No. 1 cc.	2	2	2	2	2	2	2	2	2	2	2	2
Final starch concentration	1·078	0·647	0·431	0·216	0·129	0·0863	0·0500	0·0400	0·0300	0·015	0·005	0·005

The first point which becomes evident from this set of data is that there is no evidence of a "critical" starch concentration for the occurrence of the reaction as has been suggested by Eadie [1926]. Even in the digests of lowest

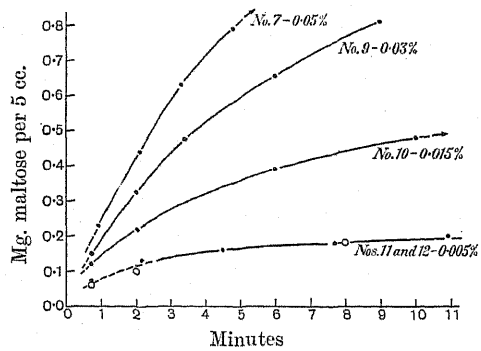


Fig. 1. Progress curves of digests of low starch concentration, from 0·050 % to 0·005 %.

starch concentration (0·005 %) the reducing power increases progressively during the period under observation. This is shown clearly in Fig. 1 in which progress curves of several digests of low substrate concentration have been plotted[2].

[1] This amylase preparation showed an optimum activity between p_H 4·6 and 4·8. This has been found to be true of nearly all the preparations of barley amylase investigated. The data on the p_H-activity relationships will be discussed in a subsequent paper.

[2] Since several of the sugar determinations in this experiment fell below the range of the sugar method as originally standardised, the method was re-standardised for maltose down to 0·05 mg. The linear relationship between the amount of maltose and the ferricyanide reduced was found to hold over the entire range investigated.

Table II.

Digest No.	*a* % starch concentration	*t* Mins. after zero time	*x* mg. maltose per 5 cc.	Slope mg./min.	*v*, initial slope: Earliest measured	*v*, initial slope: Extrapolated*
1	1·078	1·0	1·12	0·438	0·438	0·445
		3·1	2·04	0·408		
		5·0	2·815	0·408		
		7·0	3·63			
2	0·647	0·8	1·08	0·433	0·433	0·435
		2·6	1·86	0·424		
		4·5	2·665	0·403		
		6·2	3·35			
3	0·431	0·75	0·575	0·386	0·386	0·400
		2·5	1·25	0·372		
		4·3	1·92	0·354		
		5·9	2·485			
4	0·216	0·7	0·415	0·334	0·334	0·345
		2·3	0·95	0·308		
		3·6	1·35	0·308		
		4·9	1·75			
5	0·129	0·7	0·295	0·300	0·300	0·305
		2·0	0·685	0·269		
		3·3	1·035	0·232		
		4·7	1·36			
6	0·0863	0·7	0·25	0·238	0·238	0·260
		1·9	0·535	0·221		
		3·1	0·80	0·196		
		4·3	1·035			
7	0·0500	0·9	0·23	0·175	0·175	0·180
		2·1	0·44	0·158		
		3·3	0·63	0·107		
		4·8	0·79	0·085		
		10·1	1·24			
8	0·0400	0·7	0·155	0·154	0·154	0·165
		1·9	0·34	0·145		
		3·0	0·50	0·113		
		4·1	0·625			
9	0·0300	0·7	0·15	0·135	0·135	0·140
		2·0	0·325	0·107		
		3·4	0·475	0·069		
		6·0	0·655	0·052		
		9·0	0·81			
10	0·015	0·7	0·12	0·077	—	—
		2·0	0·22	0·045		
		6·0	0·39	0·028		
		10·0	0·48	0·005		
		23·0	0·55			
11	0·005	0·7	0·07	0·040	—	—
		2·2	0·13	0·013		
		4·5	0·16	0·006		
		7·7	0·18	0·006		
		10·9	0·20			
12	0·005	0·7	0·06	0·031	—	—
		2·0	0·10	0·013		
		8·0	0·18			

* The method of extrapolation is described on p. 1409. The values are given to the nearest 5 in the third decimal place. This implies a significance ranging from about 0·5 % in the highest values to about 2 % in the lowest.

Digests 10, 11 and 12 were carried out primarily to determine whether or not hydrolysis occurred and no attempt has been made to measure reaction velocities with such low starch concentrations.

The initial sugar values, calculated from separate determinations of the reducing power of the enzyme and the substrate, were, for digest 10, 0·04 mg.; for digests 11 and 12, 0·025 mg., expressed as maltose per 5 cc. Knowing these values it is possible to calculate the extent of the reaction in these digests. In digest 10 (0·015 %), approximately 59 % of the starch is hydrolysed at 10 minutes; 70 % at 23 minutes. In digests 11 and 12 (0·005 %) 72 % of the starch is hydrolysed at 10 minutes. It is clear that the reaction proceeds to the normal extent in these extremely low substrate concentrations.

These results, which have been confirmed using three different preparations of amylase, are contrary to the findings of Eadie who reported that no hydrolysis took place below a certain critical starch concentration, which varied in his different experiments from about 0·03 % to 0·09 %, as estimated from his graphs.

It is now proposed to consider the information obtainable from the data of digests 1–9 concerning the relationship between substrate concentration and initial reaction velocity. Progress curves of these digests are shown in Fig. 2. The curves have been made to pass through the origin by subtracting

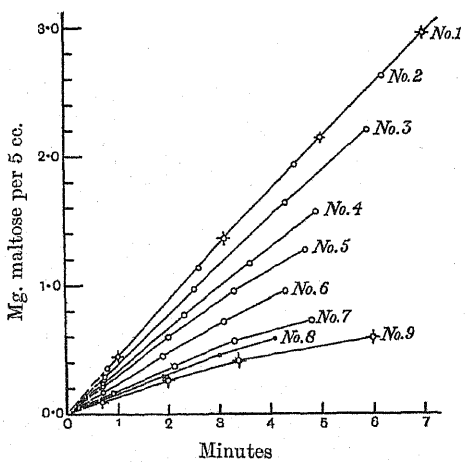


Fig. 2. Progress curves of digests 1 to 9 of substrate concentration varying from 1·078 % in No. 1 to 0·030 % in No. 9 (see Table II).

from each of the observed x values of each digest a quantity equal to the apparent initial sugar value (Sa) for that digest calculated from the earliest measured slope (*e.g.* for digest 1, $\text{Sa} = 1{\cdot}12 - 1{\cdot}0 \times \frac{2{\cdot}04 - 1{\cdot}12}{2{\cdot}1} = 0{\cdot}68$). This arbitrary procedure facilitates the visual comparison of the plotted data, and has been adopted solely for that reason.

The values of initial velocity found by extrapolation, in the manner described on p. 1409, are given in the last column of Table II. It will be seen by comparing the figures in the last two columns that the differences between the earliest measured slopes and the extrapolated values are small. Actually, the conclusions which will be drawn from the data would be almost equally valid if either set of values had been used.

The relationship predicted from the Michaelis theory is

$$v = V_\infty a/(a + K_s).$$

The values of the constants V_∞ and K_s for this expression giving the best fit to the data of digests 1 to 9 have been calculated by the method of least squares. It was assumed that the values of a, the substrate concentration, were without experimental error, as compared with the error in the determinations of v. A set of equations of the form

$$a/v = a/V_\infty + K_s/V_\infty$$

was constructed from each available pair of a and v values[1]. From these, two equations normal in respect to $1/V_\infty$ and K_s/V_∞ respectively were derived in the usual way. By solving these equations, the values of best fit were found to be[2]: $V_\infty = 0{\cdot}478$, $K_s = 0{\cdot}077$. The equation

$$v = 0{\cdot}478\, a/(a + 0{\cdot}077)$$

has accordingly been used to calculate theoretical values for comparison with the observed values of reaction velocity. These are given in Table III.

Table III.

a Starch concentration %	v calculated $v = 0{\cdot}478a/(a+0{\cdot}077)$	v observed
1·078	0·446	0·445
0·647	0·427	0·435
0·431	0·406	0·400
0·216	0·352	0·345
0·129	0·299	0·305
0·0863	0·252	0·260
0·0500	0·188	0·180
0·0400	0·163	0·165
0·0300	0·134	0·140

It is clear that the relationship is in close agreement with that predicted from the Michaelis theory. The experimental results have been plotted in Fig. 3, together with the curve of the equation $v = 0{\cdot}478\, a/(a + 0{\cdot}077)$.

Exp. 2. A second experiment, carried out in the same manner as Exp. 1, will be described briefly. The enzyme was amylase 2, prepared from germinated

[1] This method will give those values of $1/V_\infty$ and K_s/V_∞ which give the minimum value for the sum of the squares of the deviations between calculated and observed values of $1/v$ (not of v); but this is good enough for the present purposes.

[2] Owing to the fact that the molecular weight of starch is unknown, the substrate concentrations have been expressed as percentages. Consequently this K_s value is not comparable with the values obtained for other enzymes.

barley (see Appendix). The substrate in this case was prepared from air-dry soluble starch which contained approximately 4·90 % of water. A 1 % solution was made up following the same procedure as described above. Seven digests of different starch concentration were followed, these being of 29 cc. volume as before, containing 2 cc. of amylase, 2 cc. of $M/5$ acetate buffer (final p_H, 4·70–4·72) and varying proportions of the 1 % starch solution and distilled water. The data obtained from these digests, Nos. 13 to 19, are shown

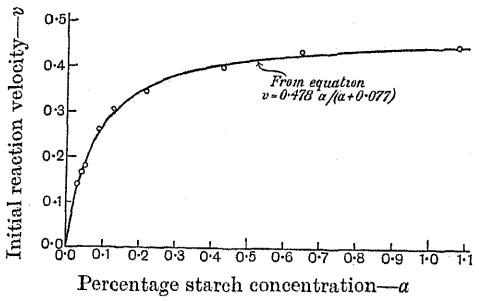


Fig. 3. The relationship between reaction velocity and substrate concentration. The values of v and a of Exp. 1 have been plotted, together with the rectangular hyperbola of best fit.

Table IV.

Digest No.	a % starch concentration	t Mins. after zero time	x Mg. maltose per 5 cc.	Slope mg./min.	v, initial slope: Earliest measured	v, initial slope: Extrapolated*
13	0·820	1·0	1·525		0·495	0·505
		3·0	2·515	0·495		
		5·0	3·49	0·487		
		7·0	4·395	0·452		
14	0·459	1·0	0·815		0·465	0·470
		3·4	1·93	0·465		
		5·6	2·94	0·459		
15	0·262	1·0	0·845		0·429	0·440
		3·4	1·875	0·429		
		5·6	2·76	0·402		
16	0·164	1·0	0·91		0·360	0·370
		3·75	1·905	0·360		
		4·75	2·255	0·350		
		7·25	3·07	0·326		
17	0·0983	1·0	0·645		0·286	0·300
		2·5	1·075	0·286		
		4·0	1·455	0·253		
		6·0	1·90	0·223		
18	0·0656	0·82	0·32		0·242	0·250
		2·08	0·625	0·242		
		3·2	0·865	0·214		
		5·0	1·23	0·202		
19	0·0328	0·8	0·255		0·154	0·165
		2·0	0·44	0·154		
		3·0	0·58	0·140		
		5·0	0·785	0·103		

* The method of extrapolation is described on p. 1409.

in Table IV, together with the values of the initial starch concentrations, corrected for the moisture content of the air-dry starch[1].

The values of best fit of K_s and V_∞ calculated for this set of data by the method which was used in Exp. 1, were found to be $V_\infty = 0{\cdot}554$, $K_s = 0{\cdot}079$. Values calculated from the equation $v = 0{\cdot}554\, a/(a + 0{\cdot}079)$, for the different substrate concentrations employed are shown in Table V.

Table V.

a %	v calculated $v = 0{\cdot}554a/(a+0{\cdot}079)$	v observed
0·820	0·506	0·505
0·459	0·473	0·470
0·262	0·426	0·440
0·164	0·374	0·370
0·0983	0·307	0·300
0·0656	0·251	0·250
0·0328	0·163	0·165

Here again the relationship is in close agreement with that predicted by the theory. Moreover it will be noticed that the value of K_s for this amylase preparation (No. 2) is the same within the limits of the experimental error, as that found for amylase 1.

It is clear from the results of these two experiments that the Michaelis theory offers a quantitative interpretation of the effects of substrate concentration upon the velocity of starch hydrolysis by the amylase of germinated barley. The evidence that the theory is applicable to this enzyme-substrate system seems at least as conclusive as that which exists for other cases, including the invertase-sucrose system.

A discussion of the effects of various factors, including hydrogen ion concentration, upon the magnitude of K_s for this enzyme will be reserved for a later paper. It should be mentioned here that other somewhat less extensive sets of data for other preparations of the enzyme indicate that the K_s value is normally close to the values which have been found for amylases 1 and 2, when the enzyme is acting at the optimum hydrogen ion concentration upon a substrate of soluble starch prepared as described above.

A discussion of the results of Eadie.

It seems necessary to discuss further the discrepancy between the results which have now been presented and the results of Eadie [1926].

Eadie came to the conclusion that with low starch concentrations no reaction occurs. His substrate concentration-velocity curves consistently show

[1] In making the correction for the water content of the starch it was unfortunately necessary to rely upon a moisture determination carried out some time after the experiment was done. The values of a and of K_s are therefore subject to an additional error not exceeding 2 %, which is absent from the results of Exp. 1. While the determination of K_s is accordingly slightly less reliable on this account, conclusions regarding the form of the substrate concentration-velocity relationship are of course not affected.

velocities of zero over a short range of low substrate concentration. Several points deducible from the curves shown by Eadie (the primary data are not published) bring the actuality of his results under suspicion. In the first place it is true in general of his data that the more active the enzyme as judged by the reaction velocity with high substrate concentration, the lower is the "critical" starch concentration required for the reaction to take place. Moreover, in the graph showing the relationship between reaction velocity and relative enzyme concentration, the points do not indicate a linear relationship, but strongly suggest that with enzyme concentrations lower than 0·4 on his scale, the reaction velocity would be zero. These points suggest that estimates of low reaction velocities by Eadie's method were unreliable.

An investigation of the technique used by Eadie has shown this to be the case. The method he used was an approach to an initial velocity method. Sugar estimations were made at 1, 5 and 10 minutes. The average rates for the 4 and 9 minute intervals were calculated and these were averaged to obtain the value which was taken as the reaction velocity. The reducing power was determined by the original micro-method of Shaffer and Hartmann [1921]. When this method was used by the writer to follow the progress of sugar production in digests of low starch concentration (0·05 %) using enzymes of low activity, comparable with those used by Eadie, it was found that the progress curves showed an initial flat phase, after which the reducing power began to increase. The duration of the initial flat phase was found to depend upon the amount of enzyme added, the longest observed being about 35 minutes. It was next demonstrated that the reducing power actually increases from the beginning, by adding a small amount of glucose to one of a pair of digests before the enzyme was added. The digest with added glucose showed an increasing reducing power from the beginning; the other showed an initial level phase.

The micro-method of Shaffer and Hartmann was then tested against known small amounts of glucose, added to the standard 5 cc. of the copper reagent. Practically no reduction was apparent until glucose in excess of about 0·1 mg. had been added. Up to 0·3 mg. the method was entirely unreliable, the values being consistently low[1].

This defect in the sugar method used by Eadie makes clear the reason for his anomalous results. When the reaction velocity in a particular digest was so small that the amount of reducing sugar produced in 10 minutes was below the critical value of the method of sugar determination, then a reaction velocity of zero was recorded. This seems to provide the explanation of the various characteristics of Eadie's results which have been mentioned.

There remains no evidence for the view that a certain critical starch

[1] Shortly after this work was done in 1926 similar conclusions as to the unreliability of the Shaffer and Hartmann micro-method with small amounts of glucose were published by Duggan and Scott [1926]. Later a modification was proposed by Somogyi [1926] to correct this defect in the original method.

concentration is necessary for starch hydrolysis by this amylase. Re-investigation of the point with the modified Hagedorn and Jensen method has shown that reducing sugar is produced even when the starch concentration is as low as 0·005 %, and that, moreover, the reaction goes to about 75 % completion, which is the normal extent of the hydrolysis of starch by barley amylase.

The effect of amylase concentration upon reaction velocity.

According to the Michaelis theory, the reaction velocity would be expected to be proportional to enzyme concentration so long as the concentration of enzyme remains small compared with that of the substrate. A number of experiments with different preparations of barley amylase have shown this to be true. A single example will serve to illustrate the experimental establishment of this relationship.

When investigating the effects of some factor other than substrate concentration, it is advantageous to employ a relatively high substrate concentration in order that the falling-off in velocity due to its transformation into products may be minimised. It has been found that the procedure for maltose estimation is somewhat facilitated if the starch concentration is not too high. For most purposes a starch concentration of 0·43 % (25 cc. of 0·5 % starch solution in a 29 cc. digest) has been found satisfactory. It will be seen from Fig. 3 that at this concentration the velocity is approaching its maximum. Occasionally for particular purposes higher starch concentrations have been used.

Exp. 3. Amylase No. 3, prepared from germinated barley, was used. Immediately before the experiment samples of the enzyme were prepared in different dilutions.

Table VI.

Digest No.	e Relative enzyme concentration	t Mins. after zero time	x Mg. maltose per 5 cc.	Slope mg./min.	v, initial velocity: Earliest measured	v, initial velocity: Extrapolated*	Ratio v/e
20	100	0·7	1·40	1·325	1·325	1·35	0·0135
		1·9	2·99	1·28			
		2·9	4·27				
21	75	0·6	1·11	0·993	0·993	1·01	0·0135
		1·9	2·40	0·964			
		3·0	3·46				
22	50	0·7	0·88	0·684	0·684	—	0·0137
		2·9	2·385	0·680			
		4·2	3·27				
23	37·5	0·6	0·64	0·487	0·487	0·495	0·0132
		2·1	1·37	0·480			
		3·5	2·04				
24	25	0·6	0·53	0·352	0·352	—	0·0141
		2·9	1·34	0·350			
		4·1	1·76				
25	12·5	0·75	0·415	0·166	0·166	0·167	0·0134
		3·0	0·79	0·160			
		5·0	1·11				

* The method of extrapolation is described on p. 1409.

Digests were made up of 25 cc. of 0·5 % soluble starch, 2 cc. $M/5$ acetate buffer (final p_H 4·73) and finally 2 cc. of amylase (of different strengths in the different digests). The reaction temperature was 25·0°.

The data obtained from these digests are given in Table VI.

The values of the ratio v/e, shown in the last column of Table VI, are approximately constant. The experimental data have been plotted in Fig. 4 together with the straight line of best fit, whose equation was found by the method of least squares to be $v = 0{\cdot}001345\,e + 0{\cdot}006$. The close distribution of the points about this line is good evidence that the relationship is linear.

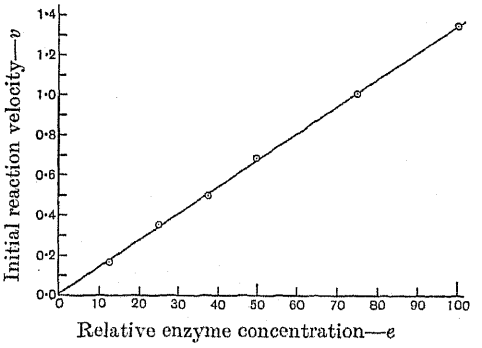


Fig. 4. The relationship between reaction velocity and enzyme concentration.

In a similar experiment carried out with an enzyme of considerably lower activity, the relationship was examined over a lower range of reaction velocities. The reaction velocities varied from 0·36 with the highest amylase concentration ($e = 16$) down to 0·022 with the lowest ($e = 1$). Here again there was no doubt that the relationship was linear, although the experimental error in the measurement of very low reaction velocities is noticeably greater[1].

It can be concluded from these experiments that the initial reaction velocity (over the range 1·35 to 0·022) is proportional to the concentration of amylase.

Summary.

An "initial slope" method of measuring the velocity of starch hydrolysis in the presence of amylase is described.

The effect of starch concentration upon reaction velocity has been investigated, using the amylase of germinated barley. The observed relationship is in close agreement with that predicted by the Michaelis theory.

[1] It has been repeatedly observed that the reaction velocity falls off more rapidly in digests containing very low concentrations of amylase than in digests containing greater concentrations of the same enzyme preparations. Since this cannot be due to falling substrate concentration, it suggests that the enzyme at high dilution in the presence of the substrate becomes subject to an inactivation or inhibition. It is impossible at the present time to indicate the possible nature of this effect.

The relationship between initial reaction velocity and enzyme concentration has been found to be linear over a wide range of enzyme concentration.

It is a pleasure to acknowledge my indebtedness to Mr G. E. Briggs and to Dr F. F. Blackman for much helpful criticism and advice.

Appendix.

Details of amylase preparations.

Amylase 1. 100 g. Spratt-Archer barley of good malting quality, harvested 2 years earlier near Cambridge. Soaked—10 hours. Germination—4 days at 17–20°. Ground and extracted for 6 hours with 600 cc. of 0·1 % acetic acid at 3°. Precipitated with 70 % (by volume) ethyl alcohol, and allowed to stand 12 hours at 3°. Filtered off precipitate, and made up to 2000 cc. Solid content of preparation, 17 mg. per 100 cc.

Amylase 2. 100 g. Plumage-Archer barley of excellent malting quality, harvested 12 months earlier at Norwich. Soaked—10 hours. Germination—6 days at 20–22°. Extracted for 48 hours with 500 cc. of 0·1 % acetic acid at room temperature. Filtered and precipitated with 50 % alcohol. Filtered off precipitate after 18 hours, and made up to 500 cc. Solid content not determined.

Amylase 3. 500 g. Spratt-Archer barley of fair malting quality, harvested 3 months earlier near Cambridge. Soaked—12 hours. Germination—4 days at 20–22°. Extracted for 24 hours with 1 litre of 0·1 % acetic acid at room temperature. Precipitated with 65 % alcohol. Filtered and made up to 1000 cc. Solid content not determined.

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CLXVIII. A NOTE ON THE CALCIUM CONTENT OF CABBAGE.

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During some experiments on the calcium balances of rabbits receiving a small daily allowance of cabbage it was considered advisable to determine the amount of calcium in the leaves taken from different parts of the plant. The variations found were so large that even when as little as 10 g. of cabbage a day were given and the rest of the daily diet contained 150 mg. of calcium, calculations of the balances might be completely fallacious if leaves from one part of the plant were taken for analysis and leaves from a slightly different position in the plant were given to the animals to eat. Sherman [1928] draws a distinction between the calcium content of cabbage, which he gives as 45 mg. per 100 g., and that of cabbage greens, which is given as 106 mg. per 100 g., but none of the food analysis tables in common use seem to mention the extraordinarily high calcium values which may be found in the outermost leaves of the cabbage.

Methods.

Cabbages were obtained from Covent Garden Market during the month of June. Samples of the leaf from different parts of the plant were weighed moist and ashed in silica crucibles, the ashing being completed with the aid of HNO_3. The white ash was dissolved in dilute HCl and the calcium in the solution was estimated by the method of McCrudden [1911]. On one or two occasions this method was checked by the method of Aron [1907], as modified by Plimmer and Page [1913], which consists of weighing the $CaSO_4$ precipitated from the filtrate of the ash solution by H_2SO_4 and absolute alcohol.

Results.

From these results it is apparent that as small a daily ration of cabbage as 10 g. may supply anything up to 100 mg. of calcium and there may be a fivefold variation in the calcium contents of leaves from the same plant which are indistinguishable in appearance, as in the last experiment quoted. It has been claimed by Culhane [1927, 1930] and again more recently by Dupré and Semeonoff [1931–32] that feeding cabbage to rabbits raises the concentration of calcium in their serum, while Kapsinow and Underhill [1929] denied

Table I.

Exp. No.	Description of leaf	Calcium: mg. per 100 g. moist leaf
1	Outer dark green leaf	476
	Inner pale green leaf	35
2	Outer dark green leaf	910
	Inner yellowish heart leaf	34
3	Outer dark green leaf	998
	Inner pale green leaf	53
4	Outermost leaf, dark green	708
	Third leaf from outside, pale green	96
	Yellowish white heart leaf	26
5	Outermost leaf, dark green	1058
	Third leaf from outside, dark green	216
	Inner leaf, pale greenish yellow	71
	Heart leaf, yellowish white	32

that cabbage had such an effect. It would seem possible that this discrepancy might be due to considerable variations in the calcium content of the cabbage used by the different observers. Incidentally the outermost leaves of the particular cabbages used for the above estimations are richer sources of calcium than any other common food, with the possible exception of cheese, and though such leaves are discarded by man they are usually preferred by the rabbit.

SUMMARY.

The calcium content of the outermost leaves of the cabbage in summer may be from 20 to 30 times as great as that of the inner leaves.

Grateful acknowledgment is made of a grant from the Medical Research Council, part of which was used to defray the cost of this work.

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CLXIX. STUDIES ON THE MINERAL METABOLISM DURING PREGNANCY AND LACTATION. II.

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(*Received July 11th, 1932.*)

From metabolism studies performed by us earlier [1931] it was evident that pregnant women on an unregulated diet often showed negative Ca and P balances during the last part of pregnancy. The diet examined contained 0·5–1·0 g. Ca and about 1·0 g. P daily and had a low content of vitamins A and D. This negative balance could be made positive or less negative by increasing the calcium content of the diet to 1·6–2·0 g. daily. In the majority of experiments cod-liver oil or a vitamin D preparation (vigantol) did not affect the negative balance when the Ca and P intake was low. If the Ca and P intake was fairly high, vitamin addition might in some instances change the balance to a positive one.

These studies led us to perform some metabolism experiments on dogs during gestation in order to follow the effect of such a diet low in Ca, P and fat-soluble vitamins on the mother and the young. The result was that the mother animal on a diet containing about 10 mg. Ca and 16–18 mg. P per kg. of body weight lost about 10 % of her original Ca content and developed a marked osteomalacia during gestation and lactation. During lactation manifest symptoms of tetany developed. The young got marked rickets with inability to walk.

As the Ca and P deficiency according to our experiments in women seemed to be the decisive factor in the negative salt balance, a parallel experiment on dogs was performed simultaneously in order to follow in detail the effect of a pure salt deficiency on mother and young.

The result was development of osteoporosis in all the young with poor formation of osteoid tissue in one of the puppies. As was the case with the first experimental litter, these puppies were also in a poor condition, they were unable to walk and could hardly move on account of multiple fractures of the hind legs.

In both these experimental litters the chemical changes taking place in the bony system of the new-born puppies were about the same, *i.e.* a distinct lowering was found in the Ca and P contents of the ash of the bones.

We wanted now to study more thoroughly this congenital osteoporosis found in all puppies by repeating the last experiment of a pure salt deficiency during gestation, and then following the development of these puppies under various nutritional conditions in post-natal life.

As in previous experiments a one year-old bitch was given a ration low in Ca and P, consisting of wheat, rye flour and cocoa fat cooked to a gruel, to this were added dried skimmed milk, raw horse meat, marmite, NaCl, Fe, lemon or orange juice and cod-liver oil.

The daily ration consisted of:

Meat	50 g.
Milk powder	10 g.
Cocoa fat	10–40 g.
Marmite	5 g.
NaCl	3 g.
Fe	0·1 g.
Lemon juice	5 cc.
Cod-liver oil	10 cc.

To this were added wheat and rye flour from 90 to 360 g. daily (according to the appetite of the dog). The Ca and P content of this diet calculated per kg. of body weight was somewhat higher in this experiment than in our earlier experiments as the dog had a lower weight. The Ca and P content ranged from 14 to 17 mg. Ca and from 37 to 40 mg. P per kg. of body weight. The Ca/P ratio was 0·4–0·5. The dog was kept in a large room with no free access to direct sunlight. A 3 days metabolism experiment was performed for Ca, P and Mg every week throughout the whole gestation period.

From Table I it is evident that with the exception of the two first metabolism periods all the remaining 7 balances were negative as far as Ca is concerned. The same may be said about phosphorus. Only during the 2nd and 8th weeks of gestation did the animal show a positive P balance. In all other experiments the P followed the Ca. The Mg balance was positive throughout the whole gestation period.

As far as the blood analyses are concerned serum-Ca (see Table I A) showed a slight lowering during the 4th, 5th, 6th and 7th weeks of gestation compared with the value of 12 mg. in the very beginning of gestation. These figures are however within the normal range. As to the P content of the serum there is a definite and constant lowering from 5 mg. in the 1st week to the lowest value of 2·9 mg. in the 9th week of gestation.

After the normal gestation period of 9 weeks the bitch gave birth to two normal-looking puppies. As the litter was so small, we were not able to perform any analyses of the new-born puppies, as were done in previous experiments. Both puppies were allowed to stay with the mother and suckled for 5 weeks. They were then given the same ration as the mother received during gestation and lactation, save that one of the puppies, Mosse, got a standardised and sufficient dose of cod-liver oil from the day of weaning.

Table I. *Metabolism studies of the dog Tulla.*

Week of gestation	Minerals studied	Intake g.	Daily output: Urine g.	Faeces g.	Milk g.	Total g.	Balance g.	Per 100 cc. serum: Ca mg.	P mg.
A. Gestation:									
1	Ca	0·1863	0·0287	0·1576	—	0·1863	0·0000	12·0	5·0
	P	0·4720	0·4200	0·1483	—	0·5683	−0·0963		
	Mg	0·1449	—	—	—	—	—		
2	Ca	0·2059	0·0267	0·1400	—	0·1667	+0·0392	11·7	4·9
	P	0·5846	0·3683	0·1415	—	0·5098	+0·0748		
	Mg	0·1923	0·0409	0·1014	—	0·1423	+0·0500		
3	Ca	0·1969	0·0184	0·3110	—	0·3294	−0·1325	11·4	4·9
	P	0·5333	0·4244	0·2416	—	0·6660	−0·1327		
	Mg	0·1706	0·0380	0·0760	—	0·1140	+0·0566		
4	Ca	0·1897	0·0190	0·2234	—	0·2424	−0·0527	10·8	4·2
	P	0·4924	0·4010	0·1941	—	0·5951	−0·1027		
	Mg	0·1534	0·0419	0·0879	—	0·1298	+0·0236		
5	Ca	0·1871	0·0127	0·2202	—	0·2329	−0·0458	10·9	3·4
	P	0·4772	0·4497	0·1863	—	0·6360	−0·1588		
	Mg	0·1470	0·0293	0·0622	—	0·0915	+0·0555		
6	Ca	0·1988	0·0130	0·2601	—	0·2731	−0·0743	10·8	3·2
	P	0·5443	0·4047	0·1984	—	0·6031	−0·0588		
	Mg	0·1752	0·0333	0·0914	—	0·1247	+0·0505		
7	Ca	0·2063	0·0137	0·2698	—	0·2835	−0·0772	10·8	3·4
	P	0·5873	0·4256	0·2108	—	0·6364	−0·0491		
	Mg	0·1932	0·0412	0·0957	—	0·1369	+0·0563		
8	Ca	0·2097	0·0276	0·2276	—	0·2552	−0·0455	11·2	2·9
	P	0·6066	0·4152	0·1772	—	0·5924	+0·0142		
	Mg	0·2013	0·0450	0·1301	—	0·1751	+0·0262		
9	Ca	0·2022	0·0272	0·1938	—	0·2210	−0·0188	11·1	2·9
	P	0·5637	0·4427	0·1985	—	0·6412	−0·0775		
	Mg	0·1833	0·0479	0·0980	—	0·1459	+0·0374		
B. Lactation:									
3	Ca	0·1933	0·0222	0·1159	0·3760	0·5141	−0·3208	10·4	3·8
	P	0·5126	0·2331	0·2222	0·2891	0·7444	−0·2318		
	Mg	0·1619	0·0345	0·1055	0·0196	0·1596	+0·0023		
3½	Ca	0·2153	0·0205	0·0659	0·5320	0·6184	−0·4031	10·6	3·9
	P	0·6389	0·1550	0·2365	0·3804	0·7719	−0·1330		
	Mg	0·2148	0·0258	0·0954	0·0206	0·1418	+0·0730		
4½	Ca	0·2270	0·0401	0·1022	0·6636	0·8059	−0·5789	10·4	3·4
	P	0·7056	0·0556	0·3460	0·5796	0·9817	−0·2761		
	Mg	0·2428	0·0476	0·1480	0·0308	0·2264	+0·0164		
After weaning.									
1	Ca	0·2270	0·0186	0·0895	—	0·1081	+0·1189	10·9	4·0
	P	0·7056	0·1746	0·3336	—	0·5082	+0·1974		
	Mg	0·2428	0·0263	0·1372	—	0·1635	+0·0793		

Three weeks after delivery a metabolism experiment was again performed on the bitch showing an increased negative Ca balance. This negative Ca balance was about 3 times as great as the highest negative balance during gestation (3rd week). Two other metabolism experiments performed during lactation, 3½ and 4½ weeks after delivery, showed even higher negative Ca balances with a daily loss of about 0·5 g. or more of Ca (Table I B). The serum-Ca showed values below that in the beginning of gestation. The same is also true of phosphorus.

The dog looked perfectly normal throughout the whole gestation and lactation period.

Analyses of the milk from the bitch gave the lowest value of Ca 18 days after delivery. All milk analyses gave rather low figures compared with milk analyses from previously examined dogs. 30 days after delivery the analyses showed an increase in the calcium figure up to 0·237 % (see Table II).

Table II. *Analyses of milk from Tulla.*

	Per 100 g. milk		
Days after delivery	Ca g.	P g.	Mg g.
13	0·213	0·246	0·009
18	0·173	0·133	0·009
25	0·207	0·148	0·008
30	0·237	0·207	0·011

When we consider the status of this dog (see Table III) we find that the calcium loss is decidedly greater in the lactation than in the gestation period. This is evidently of common occurrence in animals. Goss and Schmidt [1930] thus found a negative Ca balance in all lactating rats. The losses for full-sized litters were as great as 20 % of the entire body content of these elements in the mother.

Table III. *Status of the dog Tulla at the end of gestation and at the end of lactation.*

	Ca g.	P g.	Mg g.
Total intake during gestation	12·4803	34·0298	10·9284
Loss through urine and faeces	15·3335	38·1381	8·4175
Loss through two foetuses	4·7072	2·9048	0·1592
Total loss during gestation	20·0407	41·0429	8·5767
Balance at the end of gestation	−7·5604	−7·0131	+2·3517
Intake through food during lactation	7·4175	20·4818	6·9299
Intake through faeces from the puppies during lactation	1·9850	1·3650	0·4200
Total intake during lactation	9·4025	21·8468	7·3499
Loss through urine and faeces	4·8110	15·5449	5·5764
Loss through secreted milk	17·3236	13·9683	0·8328
Total loss during lactation	22·1346	29·5132	6·4092
Balance at the end of lactation not counting gestation	−12·7321	−7·6664	+0·9407
Balance at the end of lactation counting gestation ...	−20·2925	−14·6795	+3·2924

For the gestation and lactation period together a loss of about 20 g. Ca, 15 g. P and a Mg retention of about 3 g. were found in this dog. On account of the very small litter we had to use the average figures from our other experiments to estimate the Ca content of the new-born puppies. Furthermore, we had to calculate approximately the mineral intake which the mother may have received through eating the faeces of the puppies. The other figures, however, are arrived at by exact analyses of urine, faeces and milk. The great Ca loss during lactation is mostly due to the high output through the milk,

while the loss through urine and faeces is about half what it had been in earlier experiments. The final result of the gestation and lactation periods is a highly negative Ca and P balance, not as high however as in our first experimental dog [1931]. The calcium loss may be estimated at about 7 % of the original Ca content of the dog.

As stated before, the bitch gave birth to two well-developed and normal-looking female puppies with birth weights of 392 g. and 383 g. respectively. These puppies were allowed to feed at the breast for 5 weeks and were afterwards given the same diet as their mother, save that one puppy was deprived of the cod-liver oil.

The puppies developed quite normally during the lactation period and showed a good weight curve. When 50 days old however, the puppy Åse (with no cod-liver oil added to the diet after weaning) showed symptoms of what usually is called rickets. The clinical evidence of rickets appeared in this instance even 5 days earlier than in a puppy, Eva, described in our earlier experiments, where salt and fat-soluble vitamin deficiencies were present during foetal life and lactation as well as after weaning. This is evident from Fig. 1. Here we also see that the weight is somewhat higher than that of Eva.

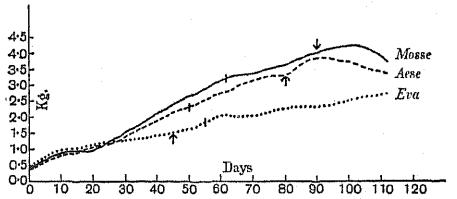


Fig. 1. Weight curves of the 3 experimental puppies.

Mosse: deficiency: foetal life: Ca and P—lactating period: Ca and P—after weaning: Ca and P.
Åse: deficiency: foetal life: Ca and P—lactating period: Ca and P—after weaning: Ca, P, vit. A + D.
Eva: deficiency: foetal life: Ca, P, vit. A + D—lactating period: Ca, P, vit. A + D—after weaning: Ca, P, vit. A + D.

↑ indicates distinct lowering of serum-P.
| indicates clinical symptoms of a pathological condition of the bones.

Thus we cannot say that the cod-liver oil given to the bitch in the present experiment, at least when there is a deficiency of Ca and P in the diet throughout gestation and lactation, has protected her puppy Åse in any considerable way against the development of the rachitic process. The rachitic symptoms: bowing of the legs, enlargement of the costochondral junctions, development of the plantigrade condition so typical in these puppies, increased constantly so that the dog had a typical rachitic appearance at about 3 months of age, when the photograph reproduced in Plate V, fig. 1, was taken.

The Ca and P analyses of the blood showed definite decreases until they reached their lowest values of 6·4 mg. Ca and 4·6 mg. P when 105 days old (see Table IV). The usual roentgenological findings typical for rickets may be seen from Fig. 2, an X-ray picture taken when the puppy was 3½ months old.

The roentgenological changes were noticed after the blood changes and the clinical symptoms considered typical for rickets had appeared.

Metabolism studies performed every week (from the 9th week) in 3-day periods showed a positive balance for all experiments with the exception of the 7th experiment as far as P is concerned (see Table IV). The daily re-

Table IV. *Metabolism experiments of the puppy Åse.*

Ca and P deficiency during foetal life and lactating period.
Ca, P and vitamin A and D deficiencies after weaning.

Age in weeks	Minerals studied	Intake g.	Daily output: Urine g.	Daily output: Faeces g.	Daily output: Total g.	Balance g.	Per 100 cc. serum: Ca mg.	Per 100 cc. serum: P mg.
9	Ca	0·0882	0·0018	0·0188	0·0206	+0·0676		
	P	0·2355	0·1364	0·0593	0·1957	+0·0398	11·0	7·4
	Mg	0·0603	0·0238	0·0354	0·0592	+0·0011		
10	Ca	0·0928	0·0300	0·0311	0·0611	+0·0317		
	P	0·2619	0·1418	0·0712	0·2130	+0·0489	12·1	8·0
	Mg	0·0714	0·0243	0·0333	0·0576	+0·0138		
11	Ca	0·0932	0·0080	0·0417	0·0497	+0·0435		
	P	0·2641	0·1546	0·0695	0·2241	+0·0400	9·2	8·0
	Mg	0·0724	0·0263	0·0367	0·0630	+0·0094		
12	Ca	0·0878	0·0086	0·0185	0·0271	+0·0607		
	P	0·2333	0·1505	0·0384	0·1889	+0·0444	8·5	6·4
	Mg	0·0594	0·0153	0·0199	0·0352	+0·0242		
13	Ca	0·0926	0·0106	0·0197	0·0303	+0·0623		
	P	0·2608	0·1901	0·0354	0·2255	+0·0353	8·2	5·6
	Mg	0·0710	0·0260	0·0166	0·0426	+0·0284		
14	Ca	0·0909	0·0077	0·0606	0·0683	+0·0226		
	P	0·2508	0·1458	0·0995	0·2453	+0·0055	8·2	5·2
	Mg	0·0668	0·0158	0·0263	0·0421	+0·0247		
15	Ca	0·0876	0·0119	0·0566	0·0685	+0·0191		
	P	0·2322	0·2017	0·0661	0·2678	−0·0356	6·4	4·6
	Mg	0·0590	0·0239	0·0156	0·0395	+0·0195		

tention, however, for Ca as well as for P, was very low, and insufficient for a growing animal. The weight increased until the last week when it dropped about 500 g. The general condition of the dog was fairly good, it was running about, had a good appetite and wanted to play.

The puppy was killed when 112 days old, as we had done with our previously examined puppies. The radius, a rib and the lower jaw were taken out for chemical and histological examination. As far as the chemical analysis of the radius is concerned a distinct lowering was found in nearly all figures for Ca, P and Mg, see Table V. For comparison with our earlier experiments, we have placed the figures for both these last two puppies in Table V together with the figures for bone analyses of the other experimental puppies all killed at the age of 112 days, and all with different dietary deficiencies throughout the foetal life, lactation period and after weaning.

It is evident from Table V that this last experimental puppy Åse in its bone composition was in a worse condition than Siss, a puppy with no dietary deficiency during foetal life and lactation period. The mineral deficiency

Table V. *Analyses of radius from 112 days-old puppies.*

Name of puppy	Dietary deficiency of: Mother during gestation	Mother during lactation	Puppy after lactation period	Fresh wt. g.	Dry wt. g.	% ash of fresh wt.	% Ca of fresh wt.	% P of fresh wt.	% Mg of fresh wt.
Lisken (normal)	None	None	None	9·733	4·709	22·5	8·9	4·0	0·15
Siss	None	None	Ca, P and vit. A+D	14·150	5·445	15·6	6·2	2·5	0·08
% difference from normal						−30·7	−30·2	−37·5	−46·70
Grete	None	Ca, P and vit. A+D	„	14·010	3·960	8·9	3·2	1·39	0·04
% difference from normal						−61·2	−64·1	−65·30	−73·30
Eva	Ca, P and vit. A+D	„	„	8·780	2·077	5·6	1·9	0·95	0·03
% difference from normal						−75·1	−78·7	−73·30	−80·00
Bobb	Ca and P	Ca and P	Ca and P	9·940	4·320	9·1	3·8	1·6	0·06
% difference from normal						−60·0	−57·6	−60·0	−60·00
Tell	„	„	„	13·200	4·695	12·1	4·8	2·0	0·06
% difference from normal						−46·2	−46·1	−50·0	−60·00
Mosse	„	„	„	6·830	2·783	13·5	4·9	2·5	0·08
% difference from normal						−40·0	−45·0	−37·5	−46·70
Åse	„	„	Ca, P and vit. A+D	8·610	2·984	9·3	3·1	1·6	0·07
% difference from normal						−58·7	−65·2	−60·0	−53·30

throughout foetal life and lactation period has thus had a definite influence on the bone composition of this puppy, Åse. The diets of these two puppies, Åse and Siss, after weaning were the same. It is the diet during foetal life and during lactation which is the sole cause of the difference between these two puppies.

The other puppy in this experiment, Mosse, was, as mentioned earlier, given exactly the same diet after weaning as the mother animal. This experiment may thus be considered as a repetition of the earlier described experiments with puppies Bobb and Tell, see Table V. This puppy also developed clinical symptoms of what is usually called rickets which progressed, and when 81 days old it developed a fracture of the tibia of the right hind leg and therefore had great difficulty in walking.

The general condition of this puppy was definitely worse than that of its sister. Plate V, fig. 1, demonstrates fairly well the bone condition of the two puppies. The bowing and the position of the legs are about the same in both animals. The enlargement of the epiphyses of the fore legs and the plantigrade condition is, however, more pronounced in Åse (with no cod-liver oil added to the diet). When 102 days old Mosse developed a complete fracture of the femur of the right hind leg with a great dislocation, which is evident from Plate V, fig. 3. From that day the puppy could not move at all and the general condition of the animal was extremely poor. It would hardly take any food for the last days before it was killed, when 112 days old.

Metabolism experiments performed in 3-day periods every week from the 9th week of age showed a positive balance of Ca and P throughout the whole

period, with the exception of a slightly negative P balance in the last experiment. The average daily retention was usually not higher than for the sister animal (see Table VI).

Table VI. *Metabolism experiments with the puppy Mosse.*

Ca and P deficiency during foetal life, lactation period and after weaning.

Age in weeks	Minerals studied	Intake g.	Daily output			Balance g.	Per 100 cc. serum	
			Urine g.	Faeces g.	Total g.		Ca mg.	P mg.
9	Ca	0·0905	0·0046	0·0216	0·0262	+0·0643		
	P	0·2487	0·1304	0·0596	0·1900	+0·0587	10·8	7·9
	Mg	0·0659	0·0196	0·0343	0·0539	+0·0120		
10	Ca	0·0951	0·0045	0·0230	0·0275	+0·0676		
	P	0·2751	0·1426	0·0593	0·2019	+0·0732	11·6	8·6
	Mg	0·0770	0·0229	0·0314	0·0543	+0·0227		
11	Ca	0·0946	0·0068	0·0480	0·0548	+0·0398		
	P	0·2722	0·1277	0·1122	0·2399	+0·0423	12·1	8·4
	Mg	0·0757	0·0263	0·0523	0·0786	−0·0029		
12	Ca	0·0943	0·0037	0·0537	0·0574	+0·0369		
	P	0·2704	0·1228	0·1370	0·2598	+0·0106	10·8	8·5
	Mg	0·0750	0·0135	0·0748	0·0883	−0·0133		
13	Ca	0·0950	0·0044	0·0371	0·0415	+0·0535		
	P	0·2742	0·1540	0·1015	0·2555	+0·0187	11·4	6·8
	Mg	0·0766	0·0172	0·0328	0·0500	+0·0266		
14	Ca	0·0916	0·0032	0·0307	0·0339	+0·0577		
	P	0·2552	0·1495	0·1038	0·2533	+0·0019	10·0	7·2
	Mg	0·0686	0·0138	0·0315	0·0453	+0·0233		
15	Ca	0·0834	0·0025	0·0119	0·0144	+0·0690		
	P	0·2077	0·1746	0·0656	0·2402	−0·0325	10·6	6·0
	Mg	0·0487	0·0131	0·0222	0·0353	+0·0134		

Blood analyses performed every week from the day of weaning did not show any lowering of the Ca. The P, however, dropped to 6 mg. per 100 cc. serum just before death.

In Plate V, fig. 3, is noticed the very poor calcification of the shaft of the femur, where the fracture occurred. No epiphyseal changes however can be noticed here.

When killed at 112 days of age, the radius was taken for chemical examination and the result of this chemical analysis may be seen from Table V. The figures indicating the bone composition are rather close to the figures of the puppy Tell, which was given the same pure salt-deficient diet during foetal life, lactation and after weaning.

The other radius, a rib and the jaw were taken for histological examination. This shows for the puppy Mosse:

(*a*) *Radius.* The bone trabeculae in the epiphysis are few and thin compared with the normal puppy Lisken [1931]. The osteoid zones are somewhat enlarged, but a little less than in the corresponding puppies Bobb and Tell [1931].

(*b*) *Costa.* The osteoid zones are about 3–4 times as broad as in the control puppy, Lisken, but a little narrower than in Tell.

(*c*) *Mandibula.* On the basis and in the alveolar septa the osteoid zones are about 4–5 times enlarged compared with the control puppy, Lisken. This is the same as in Tell. But in contra-

diction to this last-mentioned puppy, Mosse does not show any enlarged dentinoid zone or any other great disturbance in the calcification of the teeth.

The histological examination of the puppy Åse shows:

(*a*) *Radius.* The proliferating zone of the cartilage is 5–6 times as broad as in Mosse and very irregular. The bone trabeculae in the epiphysis are larger in number and rather thick. The osteoid zones in the epiphysis as well as in the diaphysis are greatly enlarged. Some of the trabeculae have not been calcified at all. The normal fatty tissue in the bone marrow has changed to a fibrous tissue.

(*b*) *Costa.* The costochondral junction is twice as broad as in Mosse. The osteoid zones are greater here than in the radius and many broad trabeculae have not been calcified at all.

(*c*) *Mandibula.* The osteoid zones in this bone are the broadest ones.

We have noticed as a common occurrence in our experimental work that the jaws among the different bones of the organism contain the broadest osteoid zones during an osteopathic process. This observation is of great clinical interest.

The calcification process of the teeth is greatly affected. The dentinoid zones are much enlarged and the calcified dentine very irregular.

These histological investigations show as far as the puppy Åse is concerned, that we have here a histological picture of what is called rickets. The other puppy, Mosse, which received a sufficient amount of cod-liver oil after weaning, also shows definite enlarged osteoid zones—especially in the ribs and jaws—considered as a typical sign of rickets. Enlargement of the proliferating cartilage zone, however, was not present.

Discussion.

This experiment was performed in order to see what effect a pure congenital osteoporosis may have on the post-natal development of the bony system under various dietetic conditions after weaning.

We have presented two parallel experiments on puppies with Ca and P deficiencies during foetal life and during the lactation period. From our earlier experiments we know that puppies born under such conditions suffer from a general congenital osteoporosis, which is probably aggravated during lactation by the rather low Ca content of the mother's breast milk. When then one puppy was deprived from the day of weaning of the addition of cod-liver oil to the diet, while the other still received this addition, the further development of these two puppies, as far as the clinical appearance is concerned, was fairly similar. Both of them became bow-legged plantigrades and developed some enlargement of the epiphyses and the costochondral junctions, this last condition being however most marked in the puppy with no cod-liver oil. On the other hand the general condition of the latter puppy was decidedly better than that of the other which developed fractures.

While the chemical analyses show a lowering of all figures for Ca, P and Mg as a common finding for both puppies, the histological pictures are somewhat different. There is no doubt that the puppy without any cod-liver oil in the diet is suffering from rickets. The other puppy has no epiphyseal changes but has enlarged osteoid zones, a histological finding which is usually considered diagnostic of a rachitic process. Otherwise the condition of the bone corresponds rather with osteoporosis. It is at least evident from this experiment that cod-liver oil, even present in sufficient amount throughout foetal

life and lactation period, cannot protect the bones from undergoing an extreme osteopathic process resulting in multiple fractures when the mineral intake is low.

This finding agrees well with the statements of many previous investigators that the D-vitamin has less influence upon the balance of Ca than upon the intermediary distribution of Ca to the epiphyses of the long bones. Thus Brown and Shohl [1930] came to the following conclusion: "Vitamin D controls calcification of the skeleton by dissolution and deposition of the bone salts. The ash content of the bones is a result of these two activities. The amount of Ca and P in the diet determines the retention of these elements. Vitamin D controls their intermediary metabolism."

In this connection Hart *et al.* [1930] found that the Ca balance was not improved in milking cows by giving large doses of vitamin D in irradiated yeast. They were sure that vitamin D was absorbed into the blood as evidenced by the enrichment of the milk in this factor. According to their opinion this points to the fact, that there may be other factors influencing the Ca balance than the Ca level and the vitamin D intake.

Marek and Wellmann [1931] have furthermore recently shown by numerous animal experiments that the development of a true rachitic process is not strictly confined to the lack of D-vitamin in the diet. They have shown that the amount and the relationship of Ca and P in the diet together with the amount of organic matter, particularly proteins, irrespective of the lack of D-vitamin are, at least in animals, of great importance for the development of a rachitic process.

Another point of interest in this dog experiment is the influence of congenital osteoporosis on the time of development of a rachitic process in a growing organism in post-natal life. It is evident from Fig. 1 that the clinical symptoms of rickets appeared at about the same time as for the animal which experienced salt and vitamin deficiencies during foetal life as well as during lactation and after weaning. It is true that the clinical symptoms were more pronounced in the last-mentioned animal, but the time of development was about the same for both of them. In this connection we have to remember that the clinical findings of osteoporosis and of rickets in growing organisms are almost impossible to differentiate. In many instances we may therefore diagnose rickets when osteoporosis is the main pathological process. The two conditions may also be so combined in one individual case, that a true clinical differentiation is impossible. The difference may be evident only on histological examination.

We hereby extend our best thanks to the former chief of the Physiological Institute of the University of Norway, Professor S. Torup, for the opportunity of performing these investigations.

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DESCRIPTION OF FIGURES IN PLATE V

Fig. 1. Photographs of the puppies Mosse and Åse at 3 months of age. To the left Mosse *with* cod-liver oil. To the right Åse *without* cod-liver oil.

Fig. 2. Radiograph of the distal end of tibia and fibula (puppy Åse) showing rachitic changes.

Fig. 3. Radiograph of femur of the right hind leg (puppy Mosse) showing a complete fracture.

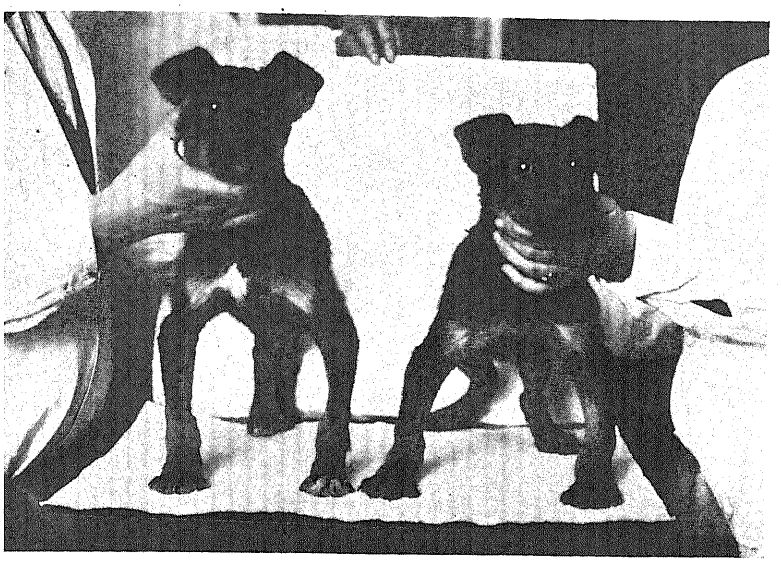


Fig. 1.

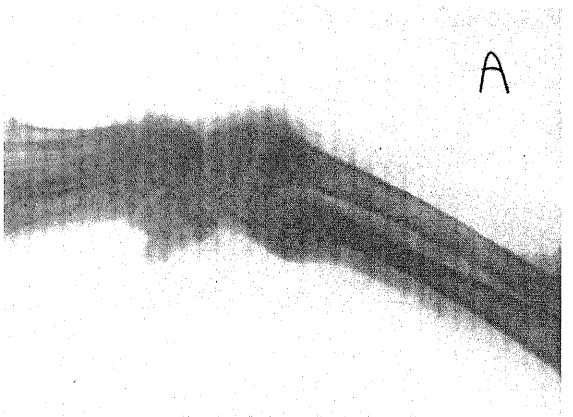


Fig. 2.

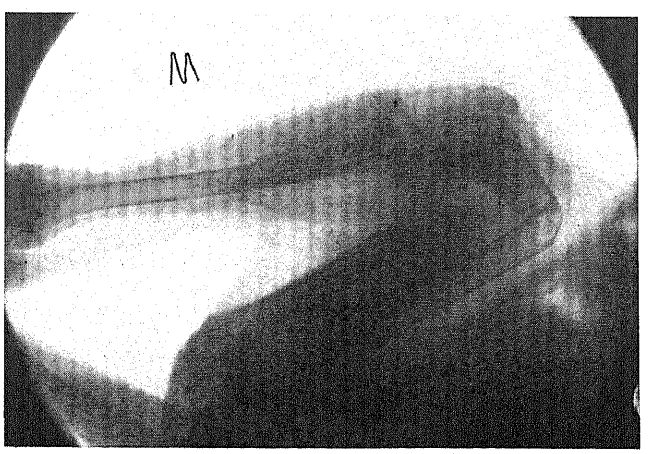


Fig. 3.

CLXX. THE RELATIVE VALUES OF THE MEALS FROM OILY AND WHITE FISH FOR GROWTH AND CALCIFICATION.

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(*Received July 13th, 1932.*)

McFarlane, Graham and Richardson have shown in a recent communication [1931], that fish meal fed at a level of 15 % of the diet is sufficient to promote normal growth in chicks: if, however, the fish meal is extracted with ether before use, poor results are obtained notwithstanding the addition of cod-liver oil to the ration.

The second statement is of great importance as it is well known that in practice it has been found necessary to extract fish meals with solvents, as an excess of oil in the meal produces taint in the flesh of animals to which it is fed.

Results obtained in experiments carried out over a period of many years prove, however, that the value of fish meals for feeding purposes is not affected by the extraction with solvents but that it depends on the quality of the raw material used and on the method of manufacture.

Already Maynard, Goldberg and Miller [1925, 1, 2], and Maynard and Miller [1927] had shown that a low calcium, high phosphorus basal ration containing linseed oil meal, produced, in growing animals, a bone very low in mineral constituents as compared with the bone developed when fish meal was substituted, on an equal protein basis, for the linseed oil meal.

The better calcification is due, according to these authors, to the presence in the fish meal of a factor aiding calcium assimilation, and this factor is contained neither in the oil fraction of the meal nor in its alcoholic extract.

These results were confirmed by Davidson [1927] who in feeding experiments on pigs found also that a fish meal prepared from so-called oily fish, from which the oil had been extracted by ether, gave a higher growth rate than a white fish meal not extracted with ether, and that the better growth was also obtained at a smaller expense of food. He came also to the conclusion that oil in fish meal appears not only to be of no special value for growth, but to have a slight depressing effect on live-weight increase [1928].

Results similar to those of Davidson were obtained by us in laboratory experiments on rats, and form the subject of the present communication.

One of the first difficulties experienced was that of obtaining, from a commercial source, products of which the raw material used and the method of manufacture were known. In another paper in course of preparation, we shall show how the latter has considerable influence on the experimental results, as commercial fish meal, and especially that prepared by the rotary-dryer method, contains highly oxidised oils which cause disturbances during the periods of reproduction and lactation.

For this reason it was decided to prepare our own meals.

Experimental.

(*a*) *Preparation of the meals.* The meals used in all the experiments were prepared by drying the fish in a small laboratory plant by means of superheated steam, thus avoiding, because of the absence of air, any danger of oxidation.

For the white meal cod and haddock were used, while for the oily meal, bream, herring or mackerel were chosen. After drying, the meals were completely extracted with ether; several batches were prepared and, after mixing, stored for use. They had the following average composition:

	Meal from white fish %	Meal from oily fish %
Crude protein (N × 6·25)	66·56	63·75
Moisture	9·02	9·68
Calcium phosphate	13·65	13·46
Fat, soluble in alcohol	0·46	0·40

(*b*) *Diet.* In preliminary experiments several diets were tried but finally it was decided to use the diet suggested by Maynard and Miller [1927], the composition of which is as follows:

Yellow cornmeal	73·5 parts
Wheat gluten	7·0 ,,
Fish meal	15·0 ,,
Calcium carbonate	2·5 ,,
Calcium phosphate	0·5 ,,

The proportions of calcium carbonate and calcium phosphate were altered when necessary in order to maintain the ratio Ca: P practically constant throughout the experiment [Karelitz and Shohl, 1927]. No sodium chloride or other salts were added as fish meal already contains sufficient.

Lemon juice and small quantities of marmite were added to the water used in mixing the food.

(*c*) *Feeding experiments.* The experiments recorded in this paper were carried out on more than 100 inbred rats, albinos and blackhoods, divided into five groups.

Three of the groups, before being fed on fish meal, were kept, immediately after weaning, on the Steenbock diet No. 2965 [1925] for a period of three weeks

or, if necessary, until the rate of growth was very small. The other two groups were, instead, fed on a vitamin-free diet (British Drug Houses) until growth had completely ceased. The rats of the first groups were apparently always in good health and very lively although small; those of the other two groups became very ill, losing their fur and showing symptoms of vitamin deficiency.

The Steenbock diet has been criticised because of the deficiency in salts and especially phosphorus; such deficiency according to Chick and Roscoe [1926] may complicate experiments with regard to vitamins A and D by setting up severe competition for phosphorus between the bones and the soft tissues. It was, however, decided to compare this diet, not wholly deficient in vitamins but deficient in mineral salts, with the other diets containing a proper balance of salts, in relation to the results obtained after feeding the animals on a diet containing fish meal.

The reason for adopting this course is that in agricultural practice young animals are fed, after weaning, on diets which although not necessarily deficient in vitamins are, practically always, deficient in the proper balance of salts.

The rats used in these experiments were fed *ad libitum*, and it was noticed that when they were transferred to the diet containing fish meal, that prepared from white fish seemed to be more palatable, as the rats on it ate more freely and grew more rapidly at the beginning. Those on the ration containing meal from oily fish soon became accustomed to it and after a week ate it freely. It was also noticed that the amount of white fish meal consumed was always greater.

The animals were weighed once a week, and the experiments continued until the growth curves showed a tendency to become flat.

There were a few instances when individual rats on a particular diet did not respond in the same way as the average animal, growing either more or less. The growth in these rare cases was not included in the results given.

In the different groups the sexes and litters were distributed as evenly as possible, but the results are given separately for males and females. Two males and two females from each group were killed at the beginning of each experiment as controls for the bone analysis, while the remainder were killed at the end of the test, the tibia and the femur being removed for the estimation of the ash.

In Figs. 1, 2, 3 and 4 are shown the average growths in g. for rats fed on white and oily fish meal after a preparatory period on the Steenbock diet, while Figs. 5 and 6 represent the growth of rats fed on the same meals but kept for a period on a vitamin-free diet.

The results obtained for the estimations of ash are given in Table I; the value A/R is the ratio of the amount of mineral substance to the amount of organic matter contained in a fat-free and moisture-free bone [Chick, Korenchevsky and Roscoe, 1926].

The results obtained in these experiments show without doubt that a fat-free meal prepared from oily fish gives a better growth rate (10–20 %) than a white fish meal, and also a better calcification of the bones.

Both meals were extracted with ether and the amount of fat, insoluble in this solvent but soluble in alcohol, was practically the same in the two meals.

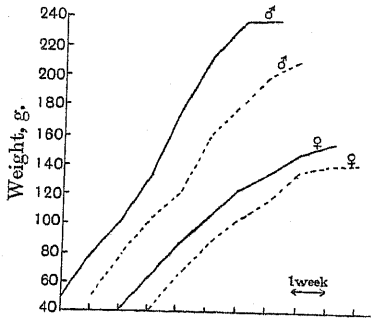


Fig. 1. Group I.

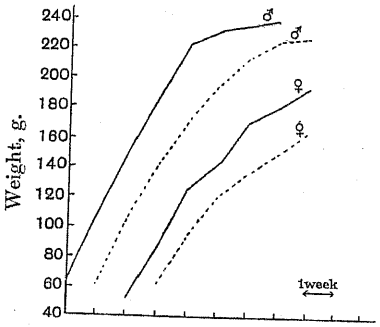


Fig. 2. Group II.

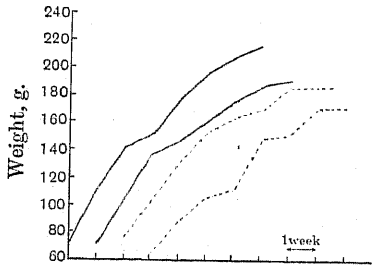


Fig. 3. Group III. (Male rats.)

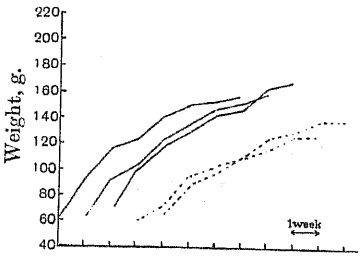


Fig. 4. Group III. (Female rats.)

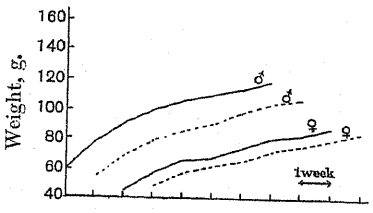


Fig. 5. Group IV.

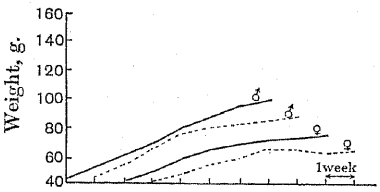


Fig. 6. Group V.

——— Oily fish meal.

– – – – White fish meal.

Table I.

	Group I (Albinos)		Group II (Blackhoods)		Group III (Blackhoods)		Group IV (Albinos)		Group V (Blackhoods)	
	Ash %	A/R	Ash %	A/R	Ash %	A/R	Ash %	A/R	Ash %	A/R
Control	43·5	0·77	43·6	0·77	—	—	43·4	0·77	38·7	0·63
Oily fish meal ♂	57·2	1·34	59·9	1·49	57·8	1·35	56·9	1·32	56·13	1·30
White fish meal ♂	52·2	1·10	58·2	1·39	56·8	1·28	55·4	1·24	55·4	1·24
Oily fish meal ♀	57·8	1·35	58·6	1·41	58·2	1·40	57·6	1·36	56·0	1·28
White fish meal ♀	53·0	1·13	58·2	1·40	55·7	1·24	56·4	1·29	54·7	1·23

It is not, therefore, possible to attribute the better growth and calcification to traces of oil left in the meal prepared from oily fish.

In the first three groups the growth is, as would be expected, considerably better owing to the ration supplied in the preparatory period. The results for calcification were, however, equally good in all the rats fed on oily fish meal, whatever the ration used in the preparatory period, proving therefore that the oily meal is richer in the necessary factor. On the other hand, the bone calcification of rats fed on white fish meal was, in many cases, below normal, whichever preliminary diet had been used.

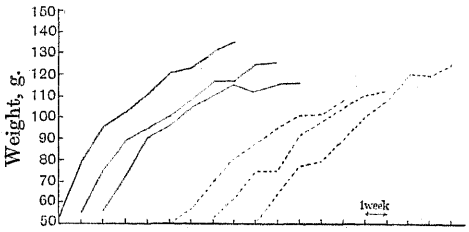


Fig. 7. Male rats.

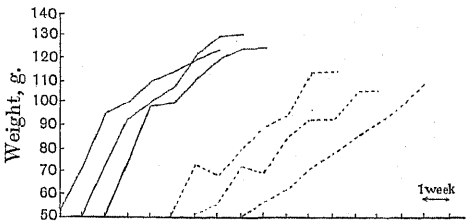


Fig. 8. Female rats.

——— Air-dried meal—9·2 % oil.
– – – – Meal dried in absence of air—1·8 % oil.

It appears, therefore, from the foregoing results that the extraction of the meal with ether does not alter its properties. These properties are however considerably modified by the methods used in the drying of the raw material as shown by the following experiments.

For purpose of comparison two commercial meals were used, prepared in different plants, but from material as far as possible of the same kind.

The meal prepared in the first plant had been partially de-oiled during the process, the drying taking place in absence of air. The oil content was 1·8 %.

The second meal was dried in a current of air and was not de-oiled, the final product containing 9·2 % of oil.

The diets used were similar to those of the previous experiments.

Seventy-five rats were fed with these two meals but as the results obtained showed very little variation the figures for one set of experiments only are given. Two litters of albino rats were used—13 females and 12 males—born on the same day. For three weeks after weaning they were kept on the Steenbock diet and then transferred to the diets containing the two fish meals.

The growth curves are given in Figs. 7 and 8.

It will be noticed that the rats fed on the second meal, containing 9·2 % of oil, showed a better growth and this was evidently due to the amount of fat present in the meal, because in these rats the adipose tissue was considerably more developed than in the rats fed on the other meal containing only 1·8 % of oil.

The calcification of the bones, however, in the heavier rats was not as good as that of the rats fed on the first diet as shown by Table II.

Table II.

	Group VI (1·8 % oil)		Group VII (9·2 % oil)	
	Ash (%)	A/R	Ash (%)	A/R
Control	41·0	0·70	—	—
♂	59·25	1·45	57·21	1·33
♀	59·06	1·44	57·24	1·34

These results confirm those of the previous experiments and show that the de-oiling does not affect bone calcification as much as the method of preparation of the meal.

It is also of interest to note that the oil which solvents would extract is a body oil, the livers and other internal organs having been removed, as a rule, from the fish immediately after it was caught. These body oils, as shown by Ahmad and Drummond [1930], are of little value for growth promotion.

Summary.

The nutritive properties of a fish meal depend largely on the kind of raw material used and the method of manufacture.

De-oiling of a meal prepared by drying so as to avoid oxidation during the process does not affect the property of the meal in aiding bone calcification.

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CLXXI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXIV. THE METABOLIC PRODUCTS OF THE *PENICILLIUM BREVI-COMPACTUM* SERIES.

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Thom, in his book "The Penicillia" [1930], groups together a number of species in what he calls the *P. brevi-compactum* series, corresponding with Biourge's [1923] Hemizonata. This series, of which the outstanding species may be taken to be *P. brevi-compactum* Dierckx and *P. stoloniferum* Thom, includes a number of other species or strains which are very widely distributed. Thom regards *P. brevi-compactum* and *P. stoloniferum* as synonyms.

The series is particularly interesting from a biochemical point of view since there is a certain amount of scattered information leading to the belief that the metabolic products of some of the species are definitely toxic. In 1896 Gosio was engaged in Italy on a study of mouldy maize in the belief that pellagra is caused by the toxic substances present in such maize. Although this belief is no longer held, Gosio's work [1896] led to the introduction of a test, which has been largely used in Italy and in America, for the detection of spoilage of maize by mould infection. This test was amplified by Black and Alsberg [1910] and consists in extracting the ground maize with chloroform. To the chloroform solution is then added ferric chloride, and in the presence of marked infections various colours such as blue, green, violet and red are obtained. Gosio isolated from mouldy maize a strain of *Penicillium* to which he gave the name *P. glaucum*, although it is evident now that his culture was inadequately named and was, in fact, one of the *brevi-compacta*. When this species was grown, either on maize itself or on Raulin's medium, a metabolic product was isolated which melted at 145°, and gave an intense blue colour with $FeCl_3$ when pure, but a violet shade when slightly impure. Unfortunately, very little of the substance was available for analysis, and hence, on results of a single combustion and without a molecular weight determination, Gosio assigned the formula $C_9H_{10}O_3$ to this product.

Alsberg and Black [1913], in a study of maize deterioration by mould infections, found that a fungus isolated from a sample of spoiled Italian maize, and identified as *Penicillium stoloniferum* Thom, is, at any rate in part, responsible for this deterioration. This organism, when grown on Raulin's medium, gave the very strong and characteristically violet ferric chloride reaction of Gosio [1896]. They isolated the metabolic product responsible for the ferric chloride reaction, and called it mycophenolic acid. This acid was obtained in the form of white needles, having a melting-point of 140°, and the formula $C_{17}H_{20}O_6$, deduced from analysis, titration value and molecular weight estimations. Alsberg and Black described its general properties, including the fact that it gives a violet colour with $FeCl_3$ in aqueous solution, but they did not work out its constitutional formula. There is, in our opinion, no doubt that Gosio's acid and Alsberg and Black's mycophenolic acid are one and the same substance. Neither of these substances was found to be toxic to mice.

Turesson [1917] found that some members of the *P. brevi-compactum* series were pathogenic to honey bees, and his experiments indicate that these species produce a phenolic substance in quantities sufficient to produce this toxic effect.

We have collected from various sources a number of species or strains of *Penicillium* belonging to the *P. brevi-compactum* series, and found that when these cultures are grown on Raulin's medium, or to a lesser degree when they are grown on a Czapek-Dox medium containing glucose as the sole source of carbon, the metabolism solution gives with ferric chloride a range of colours varying with the length of incubation and the particular species used. In the earlier stages after, say, 7 days' incubation, the colour is brown or brownish-red, and then changes with continued incubation to a port-wine red or purple, or bluish-red, or even, in some cases, bluish-violet.

Other characteristic reactions are obtained by addition to the metabolism solution of bromine water or alkaline hypochlorite solution. The cautious addition of bromine water gives first a reddish-brown colour, discharged completely on further addition of the reagent. Excess of bromine gives in most cases a voluminous precipitate. Bleaching powder or sodium hypochlorite solutions give a coloration varying from pale brown to rich brownish-red, according to the length of the period of incubation. The colour fades very slowly if the optimum amount of hypochlorite is used but is rapidly discharged by excess of the reagent.

In working up the metabolic products from a number of these species, it soon became evident that a complex mixture of metabolic products is formed. In almost all cases mycophenolic acid, in greater or smaller amount, was isolated, but in addition to this compound, other metabolic products, having the empirical formulae $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$ were isolated.

The colours given by the metabolism solution with ferric chloride or with bleaching powder solution and the reactions with bromine water are obviously the sums of the separate reactions of the various pure products with these reagents. The reactions of the pure materials are described in detail later.

It is the purpose of this paper to describe the culture methods employed for the production of these substances, and the chemical methods used for their isolation. The question of their constitution will be dealt with in subsequent papers.

Experimental.

Cultures.

The following list indicates the organisms used and their source.

Table I.

Catalogue No.	Species	Source
P 75	*P. brevi-compactum* Dierckx	Received from Centraalbureau voor Schimmelcultures, Baarn, 14. x. 30. Biourge's culture
M 3 (1)	Unnamed	Isolated at London School of Hygiene and Tropical Medicine by J. H. V. Charles from Italian mouldy maize
M 3 (3)	Unnamed	" " "
M 3 (4)	Unnamed	" " "
P 151	*P. scabrum* Biourge	From Biourge, 23. xii. 31
A 11	*P. brevi-compactum* Dierckx	Isolated from cotton and identified by G. Smith
S 30	Unnamed	Isolated at London School of Hygiene and Tropical Medicine by J. H. V. Charles from soil
P 84	*P. Hagemi* Zaleski	Type strain from Baarn, 14. x. 30
P 99	*P. Szaferi* Zaleski	Type strain from Baarn, 14. x. 30
D 8	Unnamed	Plate contaminant isolated by Dr Duncan, London School of Hygiene and Tropical Medicine
P 90	*P. patris-mei* Zaleski	Type strain from Baarn, 14. x. 30
P 37	*P. griseo-brunneum* Dierckx	From Thom, 29. iv. 30, No. 4733.68
Ad 89	*P. stoloniferum* Thom	Ardeer Collection, Thom Type 27
Ad 87	*P. Biourgeianum* Zaleski	Ardeer Collection, contaminant of culture of Alga. Identified by Thom
P 69	*P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski	Type strain, from Baarn, 14. x. 30

All the species were examined as to purity by plating out, and, where necessary, were purified by the isolation of single-spore colonies.

Detailed morphological examination showed that all the species used were true "brevi-compacta" in Thom's sense, the penicillus typical of this group being, when once recognised, easily distinguishable from all others. The named species corresponded sufficiently closely with the published diagnoses to allow of no doubt as to correct naming, but a number of them showed slight divergencies from type, particularly as regards the presence or absence of fine markings on the conidiophores and conidia. The unnamed species all corresponded very closely with the descriptions of *P. brevi-compactum* given by Biourge and of *P. stoloniferum* given by Thom—*P. brevi-compactum* is regarded by Thom as a synonym for *P. stoloniferum*—and may be regarded as strains of that species.

Media.

Three media were tried. The first (A) which we describe as "Raulin-Thom" is identical with that quoted by Thom [1930, p. 36] as due to Raulin, except for the omission of potassium silicate. This medium, however, differs from that originally described by Raulin [1869], the ammonium nitrate of Raulin's medium being replaced by ammonium tartrate. Experiments were also tried on true Raulin's solution (B), again with omission of potassium silicate. Finally, Czapek-Dox medium (C) was used in test-tube experiments. Of the three media, which have the following composition, Raulin-Thom proved the best and was used throughout the routine experiments.

Medium A (Raulin-Thom)		Medium B (Raulin)		Medium C (Czapek-Dox)	
Water	1500	Water	1500	Water	1000
*Glucose	75	*Glucose	75	Glucose	50
Tartaric acid	4	Tartaric acid	4	$NaNO_3$	2
Amm. tartrate	4	NH_4NO_3	4	KH_2PO_4	1
Amm. phosphate	0·6	Amm. phosphate	0·6	KCl	0·5
K_2CO_3	0·6	K_2CO_3	0·6	$MgSO_4, 7H_2O$	0·5
$MgCO_3$	0·4	$MgCO_3$	0·4	$FeSO_4, 7H_2O$	0·02
$(NH_4)_2SO_4$	0·25	$(NH_4)_2SO_4$	0·25		
$ZnSO_4, 7H_2O$	0·07	$ZnSO_4, 7H_2O$	0·07		
$FeSO_4, 7H_2O$	0·07	$FeSO_4, 7H_2O$	0·07		

* In each case 5 % glucose was used in place of the usual 4·6 % cane sugar (Thom) or sugar candy (Raulin).

Technique for cultivation of the micro-organisms in the routine metabolism experiments.

350 cc. quantities of the Raulin-Thom medium (A) were placed in 10l one litre conical flasks, which were plugged with cotton wool and sterilised by steaming for half an hour on each of three consecutive days. Each batch of 10l flasks was sown with a suspension in the above medium of spores prepared from two wort-agar test-tube cultures, 3 to 4 weeks old, of the desired strain. The flasks were thoroughly shaken and incubated at 24°.

Eleven of these flasks were separately removed at varying times during the incubation period, and the following analyses carried out, the contents of the remaining 90 flasks being worked up for metabolism products.

1. Determination of p_H.
2. Glucose by polarimeter.
3. Bromine absorption by Koppeschaar's method.
4. Determination of ferric chloride reaction.

The results of the first three estimations are summarised in Table II.

Change of p_H.

When the figures (Table II) for change of p_H are plotted, the curves for P 75, M 3 (1), M 3 (3), M 3 (4), P 151, A 11, S 30, P 84, P 99, D 8, P 90 and P 37 all follow approximately the same course (S-shaped) showing usually a small initial decrease in p_H from the initial value of 3·9 over the first 6 days and

Table II.

R.S.P. = % residual sugar determined by polarimeter. B.A. = bromine absorption in mg. Br per cc.

Organism	P 75			M 3 (1)			M 3 (3)			M 3 (4)			P 151		
Days	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H
0	—	—	3·9	5·27	0·11	3·9	5·48	0·14	3·9	—	—	—	4·97	0·11	3·9
1	—	—	—	—	—	—	—	—	—	5·32	0·14	3·9	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	—	—	—	3·75	0·35	3·65	3·89	0·61	3·8	3·83	0·59	3·8	3·88	0·30	3·7
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
7	2·87	1·03	3·8	2·38	1·55	3·65	2·33	1·09	3·8	2·27	0·82	3·9	2·67	0·70	3·6
8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	—	—	—	1·94	1·87	4·1	—	—	—	1·43	0·99	4·0	—	—	—
10	1·59	1·82	3·9	—	—	—	1·27	1·54	4·0	—	—	—	1·27	1·10	4·0
11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
12	—	—	—	0·97	2·18	4·4	0·78	1·70	4·4	0·67	1·36	4·6	—	—	—
13	0·84	2·28	4·3	—	—	—	—	—	—	—	—	—	0·48	1·50	4·6
14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
15	—	—	—	0·34	2·67	4·95	0·26	2·00	5·2	0·29	1·63	5·3	—	—	—
16	0·53	2·72	4·7	—	—	—	—	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	0·16	2·26	5·7	—	—	—	0·31	1·60	5·3
18	—	—	—	0·25	2·75	4·95	—	—	—	0·11	1·79	5·4	—	—	—
19	0·26	3·26	5·1	—	—	—	0·07	2·24	6·4	—	—	—	—	—	—
20	—	—	—	0·12	3·39	5·2	—	—	—	—	—	—	0·02	1·87	6·4
21	0·19	3·36	5·5	—	—	—	—	—	—	0·02	1·89	5·9	—	—	—
22	—	—	—	—	—	—	0·00	2·27	7·1	—	—	—	—	—	—
23	0·06	3·58	6·1	0·02	3·07	5·5	—	—	—	0·00	1·98	6·1	—	—	—
24	—	—	—	—	—	—	—	—	—	—	—	—	0·00	1·90	7·5
25	0·04	3·36	6·4	—	—	—	0·00	2·32	7·5	—	—	—	—	—	—
26	—	—	—	0·03	3·49	6·5	—	—	—	0·00	1·84	6·65	—	—	—
27	0·00	3·28	6·9	—	—	—	—	—	—	—	—	—	—	—	—
28	—	—	—	—	—	—	0·00	2·24	7·7	—	—	—	0·00	1·90	7·1
29	0·00	3·36	6·9	0·00	3·74	6·65	—	—	—	0·00	2·00	6·8	—	—	—
30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
31	0·00	3·15	6·9	—	—	—	—	—	—	—	—	—	0·00	2·00	7·3
32	—	—	—	0·00	3·73	7·0	—	—	—	—	—	—	—	—	—
33	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
34	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Organism	A 11			S 30			P 84			P 99			D 8		
Days	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H
0	5·32	0·08	3·9	5·03	0·11	3·9	5·13	0·11	4·0	5·27	0·10	3·9	5·27	0·14	3·9
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	4·74	0·14	3·8	—	—	—	—	—	—
4	4·00	0·24	3·7	—	—	—	—	—	—	3·80	0·48	3·3	4·46	0·26	3·7
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	—	—	—	3·31	0·96	3·85	3·02	0·26	3·65	—	—	—	—	—	—
7	2·65	0·66	3·5	—	—	—	—	—	—	2·31	1·24	3·2	2·59	0·93	3·7
8	—	—	—	—	—	—	2·01	0·35	3·8	—	—	—	—	—	—
9	1·89	1·04	3·7	1·88	1·34	3·65	—	—	—	1·43	1·74	3·2	1·68	1·76	3·7
10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
12	1·02	1·52	4·1	—	—	—	—	—	—	0·82	2·16	3·9	0·65	2·54	4·1
13	—	—	—	0·94	3·17	4·3	0·69	0·61	4·8	—	—	—	—	—	—
14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
15	0·48	1·86	4·6	—	—	—	—	—	—	0·26	2·59	4·6	0·41	2·94	4·5
16	—	—	—	0·50	3·34	4·6	0·22	0·86	5·5	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
18	0·15	2·10	5·0	—	—	—	—	—	—	0·09	2·80	5·1	0·12	2·66	5·3
19	—	—	—	0·24	3·72	5·1	—	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	0·08	1·09	6·1	—	—	—	—	—	—
21	0·11	2·24	5·4	—	—	—	—	—	—	0·02	2·86	5·9	0·03	2·51	6·6
22	—	—	—	0·09	3·74	5·6	—	—	—	—	—	—	—	—	—
23	0·03	2·32	5·6	—	—	—	0·00	1·09	6·4	0·00	3·04	6·9	0·00	2·99	6·85
24	—	—	—	0·05	3·66	6·25	—	—	—	—	—	—	—	—	—
25	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
26	0·00	2·50	6·3	—	—	—	0·00	1·02	6·3	0·00	3·03	7·25	0·00	2·48	7·4
27	—	—	—	0·00	3·87	6·8	—	—	—	—	—	—	—	—	—
28	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
29	0·00	2·50	6·7	—	—	—	0·00	1·15	6·5	0·00	2·96	7·5	0·00	2·88	7·4
30	—	—	—	0·00	4·43	7·2	—	—	—	—	—	—	—	—	—
31	—	—	—	—	—	—	0·00	1·17	6·4	—	—	—	—	—	—
32	0·00	2·46	7·0	—	—	—	—	—	—	0·00	2·88	7·6	0·00	2·11	7·7
33	—	—	—	—	3·68	7·2	—	—	—	—	—	—	—	—	—
34	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table II (*cont.*).

Organism	P 90			P 37			Ad 89			Ad 87			P 69		
Days	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H	R.S.P.	B.A.	p_H
0	5·37	0·11	3·9	4·97	0·11	3·9	5·32	0·11	3·95	5·24	0·10	3·95	5·34	0·10	4·1
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	5·06	0·34	3·8	—	—	—
4	4·06	0·34	3·9	4·02	0·34	3·4	4·58	0·42	3·7	—	—	—	5·45	0·16	3·8
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	—	—	—	—	—	—	—	—	—	3·28	0·46	3·2	—	—	—
7	2·23	0·54	3·9	2·52	0·72	3·3	2·98	1·15	3·65	—	—	—	3·66	0·32	3·0
8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	1·51	0·61	3·95	1·47	1·04	3·5	—	—	—	1·87	0·40	3·25	—	—	—
10	—	—	—	—	—	—	2·20	0·35	3·3	—	—	—	1·63	0·62	2·8
11	0·93	0·82	4·6	—	—	—	—	—	—	—	—	—	—	—	—
12	—	—	—	—	—	—	1·35	3·04	4·0	0·87	0·40	4·1	1·67	0·62	2·8
13	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
14	0·43	1·01	6·0	0·21	1·34	4·9	—	—	—	—	—	—	—	—	—
15	—	—	—	—	—	—	0·65	4·40	4·5	0·36	0·45	6·0	0·82	0·88	3·0
16	—	—	—	0·14	1·42	5·5	—	—	—	—	—	—	—	—	—
17	0·19	1·07	6·75	—	—	—	—	—	—	0·23	0·34	6·5	—	—	—
18	—	—	—	0·05	1·41	5·7	0·23	4·54	4·5	—	—	—	0·42	1·01	3·0
19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
20	0·06	1·22	7·35	—	—	—	—	—	—	0·09	0·53	7·1	—	—	—
21	—	—	—	0·02	1·44	5·9	0·09	4·49	4·6	—	—	—	0·18	1·01	3·0
22	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
23	0·02	1·22	7·6	0·00	1·82	5·7	—	—	—	0·05	0·54	7·15	—	—	—
24	—	—	—	—	—	—	0·00	4·56	4·5	—	—	—	0·03	1·12	3·0
25	0·02	1·00	7·65	—	—	—	—	—	—	—	—	—	—	—	—
26	—	—	—	—	—	—	0·00	4·68	4·5	0·02	0·62	7·25	0·00	1·23	3·6
27	—	—	—	0·00	1·68	6·5	—	—	—	—	—	—	—	—	—
28	0·06	0·98	8·2	—	—	—	—	—	—	0·00	0·56	7·9	—	—	—
29	—	—	—	—	—	—	0·00	4·77	4·5	—	—	—	0·00	1·23	—
30	—	—	—	0·00	1·84	6·7	—	—	—	—	—	—	—	—	—
31	0·06	0·91	8·4	—	—	—	—	—	—	—	—	—	—	—	—
32	—	—	—	—	—	—	0·00	4·08	4·65	—	—	—	—	1·31	—
33	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
34	—	—	—	0·00	1·84	6·6	—	—	—	—	—	—	—	—	—

then a steady rise to values varying with the different organisms between 6·6 (P 37) and 8·4 (P 90). All these organisms give varying yields of both mycophenolic acid and the products of smaller molecular weight. The curve for Ad 87 is slightly different in type, lying somewhat lower, and this organism gives no mycophenolic acid and but little of the other products. The curve for Ad 89 shows almost no change in p_H at all. This organism produces no mycophenolic acid but gives the greatest yield of the products of lower molecular weight. Finally the curve for P 69 is quite different, decreasing from the initial value of 4·1 to 2·8–3·0 and maintaining this p_H until the 24th day when a sudden rise occurs. The sugar has, however, completely disappeared before this increase in p_H is obtained. It was not found possible to obtain any of the described metabolic products from the material isolated by ether extraction of the P 69 metabolism fluid.

Change of sugar content.

The same general similarity of curves is seen when the rates of disappearance of sugar are plotted.

Bromine absorption.

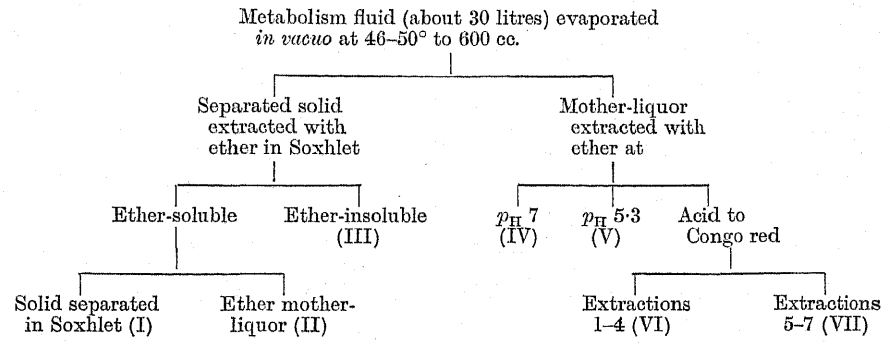
Perhaps the most interesting curves are obtained, however, by plotting the figures for the bromine absorption of the various metabolism fluids. Since the bromine absorption of fluids containing chiefly mycophenolic acid appears to be much smaller than that of those containing chiefly the products of lower molecular weight, it is possible to obtain from the bromine absorption figures some idea of the amount of total product, and of the relative amounts of the two types of products. Thus the highest bromine absorption was obtained with Ad 89, which produces no mycophenolic acid but gives the largest yield of the products of lower molecular weight. The lowest bromine absorption was obtained with Ad 87 which produced no mycophenolic acid and but little of the remaining products.

Ferric chloride tests.

The metabolism solution (5 cc.) with all the organisms except P 69, on addition of $FeCl_3$ gives initially a yellow colour, becoming dirty yellow (3rd to 4th day), pale brown (5th to 6th day), golden brown (7th to 9th day), brownish-purple (10th to 12th day), crimson (13th to 16th day), and finally a deep blue-crimson colour. With P 69, the colour change is slower and only reaches the golden brown stage. The various colours are given at slightly different times with different organisms, and the days stated must only be regarded as a general indication of the time taken.

Separation of metabolic products.

The remaining 90 flasks were removed when the sugar had disappeared and worked up in bulk for the various metabolism products. The products were first separated from the metabolism fluid in a series of fractions which were eventually examined individually. The general scheme of separation of the fractions is as follows:



The combined filtered metabolism fluid for one species (usually from 90 flasks) was evaporated *in vacuo* to 600 cc. and allowed to stand at 0° for 2 days.

(*a*) The *separated solid* was then filtered off, washed with water, dried *in vacuo*, weighed and extracted continuously with ether in a Soxhlet. A considerable amount of colourless crystalline material separated in the Soxhlet flask (= fraction I) and the ether mother-liquors on removing the ether and drying gave a semi-crystalline solid (= fraction II). The ether-insoluble material (III) consisted of salts and was not further investigated.

(*b*) The *mother-liquor* (now about 700 cc.) was then adjusted to p_H 7 (by addition of about 40–60 cc. *N* NaOH) and extracted four times with an equal volume of ether. The extracted material, after removing the ether and drying to constant weight gave fraction (IV). The aqueous mother-liquor was then adjusted to p_H 5·3 (using 20–50 cc. *N* HCl) and extracted twice with an equal volume of ether. The extracted material after removing the ether and drying gave fraction (V). Finally the aqueous mother-liquor was made acid to Congo red (by addition of 25–30 cc. conc. HCl) and extracted (*a*) four times with an equal volume of ether, the extracted material giving a red colour with $FeCl_3$ (fraction VI), (*b*) three further times with ether, the material so extracted giving a brown colour with $FeCl_3$ (fraction VII). The dry weights of all these fractions are summarised in Table III. In those cases where more than one experiment was carried out with the same organism the weights for all the experiments are added together.

Table III.

Dry weights (g.) of fractions I–VII for the various species.

Organism	No. of flasks	I	II	IV	V	VI	VII
P 75	1100	52·73	13·44	76·16	71·36	364·94	42·45
M 3 (1)	90	6·22	2·08	7·65	4·70	27·01	0·67
M 3 (3)	360	47·96	8·28	17·94	9·08	47·54	4·24
M 3 (4)	90	7·90	1·38	5·78	1·21	9·50	0·54
P 151	90	8·46	3·37	2·12	1·65	9·63	
A 11	90	6·20	1·09	3·64	—	18·50	0·55
S 30	90	4·69	4·49	4·27	2·51	34·30	
P 84	90	3·16	1·05	1·95	2·09	2·50	
P 99	90	4·02	0·87	1·50	1·77	21·25	0·95
D 8	180	0·87	0·71	10·80	2·04	32·89	0·65
P 90	90	0	0·34	3·30	1·12	2·45	1·42
P 37	90	0	1·88	2·07	1·57	12·13	0·76
Ad 89	80	0	0·62	—	—	39·35	4·80
Ad 87	90	0	1·86	—	—	2·37	0·62
P 69	90	0	1·50	6·27	—	16·09	
P 75 (Czapek-Dox) 4570 cc.	—	0	0·03	0·85	0·90	1·93	

Since 10 flasks contained 175 g. of sugar at the beginning of the experiment and the flasks were taken off when the sugar had disappeared, the yields of these fractions in relation to the sugar utilised can readily be computed from the above figures.

Isolation of products from fractions I–VII.

Fraction I consists of almost pure mycophenolic acid; fractions II and IV are grossly impure mycophenolic acid and were worked up for this product by taking advantage of the insolubility of its dipotassium salt in alcohol; fractions V, VI and VII contain only traces of mycophenolic acid, and consist of a series of products of lower molecular weight, $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$.

A. *Isolation and yield of mycophenolic acid.* In the early experiments the organism P 75 was always used and the mycophenolic acid fraction I isolated. Fraction I in all cases was practically pure mycophenolic acid, and one or two recrystallisations from boiling water or aqueous alcohol yielded a pure product, M.P. 141°, giving with ferric chloride a pure blue colour in alcohol, and a blue-violet colour in water, and corresponding exactly in properties and analysis with Alsberg and Black's mycophenolic acid. The acid obtained using the other organisms was in each case shown to be identical in properties and mixed M.P. with the P 75 product.

The mycophenolic acid of fractions II and IV was separated as the dipotassium salt. The method was tested quantitatively with a sample of P 75 fraction I. 1 g. of this fraction was dissolved in 15 cc. absolute alcohol, 6 cc. of 10 % alcoholic KOH were added and the mixture was allowed to stand 20 minutes. The precipitated dipotassium salt was filtered off, washed with absolute alcohol, dissolved in water and the free acid precipitated by addition of 2*N* H_2SO_4. After the acid had solidified, it was filtered off, washed and dried; wt. 0·95 g. Fractions II and IV in each experiment were therefore dissolved in a small amount of alcohol and the mycophenolic acid was separated in the above way. The acid so obtained after one recrystallisation from aqueous alcohol followed by one recrystallisation from boiling water was practically pure. The figures for the total yield of mycophenolic acid obtained (Table IV)

Table IV.

Organism	No. of flasks	Yield (g.) of mycophenolic acid isolated from			Total yield (g.) mycophenolic acid
		Fraction I	Fraction II	Fraction IV	
P 75	1100	52·73	7·86	11·53	72·12
M 3 (1)	90	6·22	0·49	2·67	9·38
M 3 (3)	360	47·96	1·15	4·58	53·69
M 3 (4)	90	7·90	0·34	2·10	10·34
P 151	90	8·46	0·78	Trace	9·24
A 11	90	6·20	0·29	1·05	7·54
S 30	90	4·69	1·30	1·00	6·99
P 84	90	3·16	0·55	0·93	4·64
P 99	90	4·02	0·39	0·12	4·53
D 8	180	0·87	0·10	3·83	4·80
P 90	90	0·0	0·05	2·12	2·17
P 37	90	0·0	0·98	0·80	1·78
Ad 89	80	0·0	0·0	0·0	0·0
Ad 87	90	0·0	0·0	0·0	0·0
P 69	90	0·0	0·0	0·0	0·0
P 75 (Czapek-Dox)	—	—	—	0·49	0·49

consist of the combined weights of fraction I, together with those of the weights of acid recovered from the crude fractions II and IV.

Two samples of fraction V were also worked up by the alcoholic KOH method but were shown to contain only traces of mycophenolic acid, and this fraction was therefore worked up in the same way as fractions VI and VII.

Finally a small amount of material obtained by growing P 75 on tubes of Czapek-Dox instead of Raulin-Thom's medium was worked up by the alcoholic KOH method and shown to contain mycophenolic acid.

B. *Isolation and yield of the products* $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$, $C_8H_6O_6$. The material obtained in fractions V, VI and VII by the growth of the various organisms was first dried to constant weight *in vacuo* over sulphuric acid, then finely powdered and well mixed and aliquot portions used (*a*) for preliminary estimations, and (*b*) for the isolation of the metabolic products.

(*a*) *Preliminary estimations.* These determinations included the methoxyl content, proportion insoluble in water, a rough equivalent, optical activity and a rough determination of the dry weight of the precipitate obtained with Brady's 2:4-dinitrophenylhydrazine reagent (D.N.P.). The first two estimations should give an indication of the amount of mycophenolic acid still present since this substance is the only one possessing a methoxyl group and is almost insoluble in cold water. The fraction from Ad 89, however, did contain some methoxyl, but no mycophenolic acid could be isolated.

The equivalent would only be of value if the product had contained a large amount of $C_8H_6O_6$, a condition which was never obtained. The optical activity of all fractions was always zero. The substances $C_{10}H_{10}O_5$ and $C_{10}H_{10}O_7$ give precipitates with 2:4-dinitrophenylhydrazine, whilst the products $C_{10}H_{10}O_6$ and $C_8H_6O_6$ give no such precipitate in dilute solution. Hence the weight of the D.N.P. fraction is a measure of the combined amounts of $C_{10}H_{10}O_5$ and $C_{10}H_{10}O_7$ present.

Table V. *Results of preliminary experiments.*

Organism	Fraction	Methoxyl %	Insol. in 400 parts H_2O %	Wt. D.N.P. from 1 g. of fraction and its M.P. (decomp.)
P 75 (batch 10)	VI	1·62	5·3	0·88 (199–201°)
P 75 (batches 1–8)	VII	—	3·3	0·55 (197°)
M 3 (1)	VI	1·90	3·5	0·90 (200–207°)
M 3 (3)	VI	—	6·0	0·91 (207°)
M 3 (4)	VI	—	25·0	0·68 (206°)
P 151	VI + VII	—	1·9	1·02 (202–205°)
A 11	VI	1·80	24·0	0·73 (197–203°)
S 30	VI + VII	2·40	0·0	1·03 (206–211°)
P 84	VI	—	—	(199–204°)
P 99	V + VI + VII	1·3	3·0	0·96 (202–206°)
D 8 (batches 1 + 2)	VI	1·0	0·0	0·88 (195–205°)
P 90	V + VI + VII	—	37·0	0·66 (205–207°)
P 37	VI + VII	—	4·3	1·19 (206–208°)
Ad 89	VI + VII	1·5	4·6	0·70 (200–202°)
Ad 87	VI + VII	—	—	(194–200°)
P 75 (Czapek-Dox tubes)	V + VI + VII	—	—	(190–202°)
P 75 + M 3 (3) + D 8 + P 37 + M 3 (4) + M 3 (1) + P 84 + P 151 + S 30	V	—	—	0·67 (204–206°)

(*b*) *Method of separation of the fractions of crude* $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$, $C_8H_6O_6$. The bulk (a known aliquot) of the dried fraction (see Table VI) was dissolved in purified ether (26 cc. per g.), filtered from a small amount of amorphous material and to 13 parts of the boiling ethereal solution 14 parts of boiling light petroleum (B.P. 40–50°) were added. After maintaining at the boil for a minute or two, a small amount of precipitated tar was removed by filtering through two layers of fluted filter-paper into a tared round flask. The solvent was then removed, the last traces by heating *in vacuo* for 30 minutes at 50°. The residue, a yellow or pale brown gum, was dissolved in acetone (1·2 cc. solvent per g. of gum) and to the boiling solution 55 times its volume of boiling chloroform was added, the mixture shaken and put aside to crystallise overnight. In the earlier experiments, methyl alcohol was employed instead of acetone, but the use of this solvent was later avoided as it led to partial esterification. The greyish crystalline deposit (1st crop, crude $C_{10}H_{10}O_6$) was then collected, washed with a little chloroform, weighed, and its M.P. taken.

Table VI. *Dry weights of isolated fractions and isolated crystalline substances.*

Organism	Fraction used	$C_{10}H_{10}O_5$ + $C_{10}H_{10}O_7$ g.	$C_{10}H_{10}O_6$ g.	$C_8H_6O_6$ g.	$C_{17}H_{20}O_6$ from fractions I, II, IV g.	Total isolated crystalline substances g.	Total dry wt. of fractions from which isolated g.
P 75	VI + VII	166·90	28·55	4·00	72·12	271·57	549·72
M 3 (1)	VI + VII	10·10	3·72	0·50	9·38	23·70	43·63
M 3 (3)	VI + VII	23·70	4·06	0·10	53·69	81·55	125·96
M 3 (4)	VI + VII	2·67	0·53	0·00	10·34	13·54	25·10
P 151	VI + VII	3·95	0·16	0·03	9·24	13·38	23·58
A 11	VI + VII	7·48	2·48	0·16	7·54	17·66	29·98
S 30	VI + VII	15·52	2·11	0·46	6·99	25·08	47·75
P 84	VI + VII	0·18	Trace	*	4·64	4·82	8·66
P 99	V + VI + VII	7·46	3·41	0·04	4·53	15·44	30·36
D 8	VI + VII	12·63	7·23	0·11	4·80	24·77	45·92
P 90	V + VI + VII	0·81	0·11	*	2·17	3·09	8·63
P 37	VI + VII	5·43	0·54	0·09	1·78	7·84	16·84
Ad 89	VI + VII	16·00	5·62	0·34	0·00	21·96	44·77
Ad 87	VI + VII	0·04	Trace	*	0·00	0·04	4·85
P 69	VI + VII	0·00	0·00	0·00	0·00	0·00	23·86
P 75 (Czapek-Dox)	V + VI + VII	0·23	0·25	0·00	0·49	0·97	3·71
P 75, M 3 (1), M 3 (3), M 3 (4), P 37, D 8, P 84, P 151, S 30	V	24·15	4·24	0·05	—	28·44	96·21

* In these cases the total amount of lead precipitate was so small that it would have been impossible to isolate the substance $C_8H_6O_6$ even if present.

The acetone-chloroform mother-liquor was then evaporated *in vacuo* at 50° in stages to $\frac{1}{2}$, $\frac{1}{5}$, $\frac{1}{12}$ of the original volume and finally to dryness. After each evaporation (except the last) the solution was allowed to stand 2 days for complete deposition and in this way crops 2, 3, 4 and 5 were obtained.

The second crop (relatively small) consisted mostly of $C_{10}H_{10}O_6$, and was dissolved in acetone and treated with 55 volumes of chloroform, the crystalline deposit being added to crop 1, and the residue by evaporation of the mother-liquor to crop 3. The third crop (relatively large) was then dissolved in 15 times its weight of cold water, filtering from a little tar if necessary, and *N* NaOH added (volume noted) until neutrality to litmus paper as external indicator was reached. Three times this volume of *N*/2 neutral lead acetate was then immediately added, the flask well shaken and allowed to stand overnight. The lead precipitate was then filtered off, dried *in vacuo* over sulphuric acid and weighed. The mother-liquor was acidified with dilute H_2SO_4, filtered from lead sulphate and extracted 5 times with half its volume of ether. The ethereal extract was dried over anhydrous Na_2SO_4 and evaporated at 50–60°. When distillation of ether had practically stopped, the residue which contained a considerable amount of acetic acid was poured into 50 volumes of light petroleum and the distilling flask washed out with a few cc. of ether. After standing for a few days, the precipitate (usually completely crystalline) was collected, washed with a little light petroleum, dried, weighed and its behaviour on heating carefully noted. The lead precipitate was then ground in a mortar with dilute (2*N*) sulphuric acid, filtered from lead sulphate, the filtrate extracted with ether and the ethereal extract worked up as above, the final product being usually a brownish gum. The crystalline fraction from the soluble lead salts consisted of a mixture of $C_{10}H_{10}O_7$ and $C_{10}H_{10}O_5$, and a separation was effected by fractional crystallisation from ethyl acetate-light petroleum, or chloroform-light petroleum. The gum from the lead precipitate was shown to contain the substance $C_8H_6O_6$, a considerable amount of tar, all the remaining $C_{10}H_{10}O_6$ and also amounts of $C_{10}H_{10}O_7$ and $C_{10}H_{10}O_5$, evidently carried down during the precipitation since the lead salts of the pure substances are readily soluble in cold water.

The fourth crop (relatively small) was similar in composition to crop 3 and was worked up similarly.

The fifth crop (final residue) contained nearly all the mycophenolic acid remaining, and also other tarry material insoluble in water. It was therefore extracted first with 25 times and then with 8 times its weight of cold water and the aqueous extracts filtered. The filtrates were worked up as with crop 3 and the residue on the filter was shown by crystallisation from chloroform and light petroleum to contain a small amount of mycophenolic acid.

The method was often further simplified by evaporating the acetone-chloroform mother-liquor in two stages only, first to one-fifth of the original volume and then to dryness. If the M.P. of the second crop showed it to be mostly $C_{10}H_{10}O_6$ it was added to the first crop; if the M.P. showed it to be mostly $C_{10}H_{10}O_7$, it was worked up as above.

The isolation of the substance $C_8H_6O_6$ was always difficult, partly because of the smallness in amount, but chiefly because of the impossibility of direct crystallisation from the gum recovered from the first lead precipitate. In

most instances, the material was put through a second lead acetate treatment, which led to enrichment in $C_8H_6O_6$. It was also found advantageous to use only the lead precipitate from the final residue.

The tar thrown down in the first stage of this separation was examined in the case of P 75 and shown to contain a little $C_8H_6O_6$ and $C_{10}H_{10}O_7$. It was further observed that if the crude fraction contains an abnormally large amount of $C_{10}H_{10}O_6$, the amount of acetone indicated is insufficient for complete solution, in which case more acetone is used to bring all the material into solution.

The total yield of these fractions is given in Table VI. In each case, the materials were isolated from the bulk (a known aliquot) of the fraction, and the actual figures obtained have been corrected so as to refer to the whole fraction. The weight of mycophenolic acid isolated is also given, and the total weight of isolated crystalline products is compared with the dry weight of the fractions from which they were derived.

Properties of the crystalline products.

I. *Mycophenolic acid.* This acid is almost insoluble in cold water, but crystallises beautifully in radiating needles from boiling water. Thus 5 g. of P 75 fraction I, M.P. 137–139°, on recrystallising from 3 litres of boiling water, gave 4·40 g. of dry acid, M.P. 141°. With ferric chloride it gave a pure blue colour in alcoholic solution (Alsberg and Black say a green colour) and a blue-violet colour in aqueous solution. It gives a heavy precipitate with bromine water but no reaction with bleaching powder solution. It does not reduce Fehling's solution or ammoniacal silver nitrate but is oxidised by permanganate immediately in the cold and is readily reduced catalytically by palladium and hydrogen at ordinary pressures and temperatures. It is very stable to boiling alkalis and acids. Mycophenolic acid dissolved in cold H_2SO_4 does not give a blue fluorescence in ultra-violet light. It is a very weak acid, being extracted almost completely at p_H 7, but titrates as a dibasic acid. It is optically inactive. The lead, copper and silver salts are insoluble in water and the acid may be readily separated by the insolubility of its dipotassium salt in alcohol. It contains a methoxyl group.

The following results were obtained by micro-combustion (Schoeller): C, 63·85, 63·80 %; H, 6·36, 6·33 %; OCH_3, 9·65, 9·36 %; mol. wt., 337, 341. $C_{17}H_{20}O_6$ requires C, 63·73 %; H, 6·30 %; OCH_3, 9·69 %; mol. wt., 320·2.

Also, 0·093 g. mycophenolic acid requires 5·49 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 169. (Theoretical for $C_{17}H_{20}O_6$ titrating as a dibasic acid, 160.)

II. *The substance $C_{10}H_{10}O_6$.* This substance is obtained pure by two recrystallisations from 15 parts of water of the first crop from acetone-chloroform. It forms colourless flat rhombs melting over the range 193–203° with softening, darkening and vigorous gas evolution at the latter temperature.

Micro-analysis (Schoeller): C, 52·95, 53·11 %; H, 4·72, 4·45 %; OCH_3, nil; mol. wt., 211. $C_{10}H_{10}O_6$ requires C, 53·07 %; H, 4·46 %; mol. wt., 226.

0·1057 g. required 7·5 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 141, but the end-point was very indefinite, the solution becoming brown. The substance is moderately soluble in cold ethyl and methyl alcohols and acetone, readily soluble in cold ethyl acetate, very sparingly soluble in chloroform. The lead, mercury and silver salts are sparingly soluble in water, whilst a solution of the sodium salt reduces copper sulphate in the cold in a few moments. The calcium and barium salts are readily soluble in water and the calcium salt is not precipitated by alcohol. With ferric chloride, the substance gives a stable reddish-purple colour in alcohol and a much redder colour in water, which fades in an hour.

With bromine water it gives a heavy precipitate. With concentrated sulphuric acid the substance on gently warming gives a series of colour changes from yellow-green to yellow-brown, red, deep port-wine colour, the colour being discharged on diluting with water. In dilute solution it does not give an immediate precipitate with 2:4-dinitrophenylhydrazine in 2*N* HCl. Permanganate is immediately decolorised in the cold, and bleaching powder solution gives an orange-red colour fading to yellow. The substance gives a pale green solution in cold concentrated H_2SO_4 which fluoresces blue in ultra-violet light. The colour gradually fades but the fluorescence remains.

III. *The substance $C_{10}H_{10}O_7$.* This substance along with the substance $C_{10}H_{10}O_5$ forms the fraction isolated from the soluble lead salts and is readily separated from the mixture by fractional crystallisation from ethyl acetate-light petroleum, or chloroform-light petroleum, the substance $C_{10}H_{10}O_5$ having the greater solubility. The substance $C_{10}H_{10}O_7$ melts at 125–135° with effervescence due to the loss of $1H_2O$.

Micro-analysis (Schoeller): C, 49·58, 49·51 %; H, 4·28, 4·15 %; OCH_3, nil; loss at 130–145°, 7·8 % (7·1 % H_2O, 0·7 % CO_2).

$C_{10}H_{10}O_7$ requires C, 49·57 %; H, 4·17 %; loss $1H_2O$, 7·4 %. 0·0951 g. required 7·15 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 133, but the end-point was again indefinite, the solution becoming brown. The dehydration product obtained by heating at 130–145° melts at 166–168°, but has not yet been satisfactorily obtained crystalline. When this product is crystallised from ordinary A.R. chloroform and light petroleum, it is obtained in well-defined crystals of a partially hydrated form, M.P. 166–168° (with previous softening at 116°) which analysed for $C_{10}H_8O_6$, 0·8 H_2O.

Micro-analysis (Schoeller): C, 50·27, 50·22 %; H, 4·19, 4·14 %; mol. wt., 206, 198. $C_{10}H_8O_6$ 0·8 H_2O requires C, 50·31 %; H, 4·05 %; mol. wt., 238.

When the product is crystallised from water, the original substance $C_{10}H_{10}O_7$ is obtained.

The substance $C_{10}H_{10}O_7$ is readily soluble in methyl and ethyl alcohols, ethyl acetate and acetone, fairly readily soluble in cold water, moderately soluble in cold chloroform and sparingly soluble in light petroleum. It crystallises from chloroform-light petroleum in well-formed diamond-shaped crystals. The lead salt is soluble in cold water, the silver salt sparingly soluble in cold, readily in hot water. The colour given with ferric chloride in alcoholic solution is indistinguishable from that with the substance $C_{10}H_{10}O_6$ under the same conditions, but differs from the latter in being stable and intensely purple-red in aqueous solution. The substance gives a heavy precipitate with bromine water and an immediate copious yellow crystalline precipitate with 2:4-dinitrophenylhydrazine in 2*N* HCl, but does not restore the colour to Schiff's reagent. It gives a pure, fairly stable, deep orange colour with bleaching powder solution.

With concentrated H_2SO_4 the colour reaction is very similar to that given by the substance $C_{10}H_{10}O_6$, except that it is somewhat more intense and more quickly developed on warming. Both substances give a brown colour on standing in the cold with H_2SO_4 containing a little $K_2Cr_2O_7$. With sufficient dichromate, the colour becomes green on warming. No coloration is observed, even on warming, with H_2O_2 in glacial acetic acid. The substance $C_{10}H_{10}O_7$ is more sensitive to alkali than the substance $C_{10}H_{10}O_6$. It dissolves in cold concentrated H_2SO_4 giving a pale yellow solution, but no fluorescence was obtained in ultra-violet light.

IV. *The substance $C_{10}H_{10}O_5$.* This substance can be distinguished from the substance $C_{10}H_{10}O_7$ by the fact that it first melts from 145 to 153° with effervescence and loss of water (and some CO_2), then almost immediately resets and again melts at 220–230° (decomp.), and also by its separation from water in large diamond-shaped crystals, whereas the substance $C_{10}H_{10}O_7$ separates from water in irregular aggregates of small crystals. Its separation from the substance $C_{10}H_{10}O_7$ is rendered difficult, not only by the similar solubilities of the substances and their salts, but also by the fact that a mixture containing but little $C_{10}H_{10}O_5$ behaves on melting very like the pure substance, the melt quickly resetting and remelting above 200°. The readiest separation is effected by fractional crystallisation from water.

The substance $C_{10}H_{10}O_5$ loses the elements of water on heating to give an entirely new substance $C_{10}H_8O_4$, which does not revert to the parent material on recrystallisation from water, as does the dehydration product of $C_{10}H_{10}O_7$.

Micro-analysis (Schoeller): C, 56·84, 57·28, 56·64 %; H, 4·64, 4·98, 4·68 %; loss at 150–170°, 9·4 % (7·3 % H_2O, 2·1 % CO_2).

$C_{10}H_{10}O_5$ requires C, 57·14 %; H, 4·76 %; loss of $1H_2O$, 8·6 %. 0·1703 g. required 12·75 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 134, but here again, as with the substances $C_{10}H_{10}O_7$ and $C_{10}H_{10}O_6$, the end-point was indefinite, the solution becoming brown.

With ferric chloride in aqueous or alcoholic solution, the substance behaves similarly to the substance $C_{10}H_{10}O_7$, the colour being perhaps a little more purple in shade. In alcohol, the colour with the substance $C_{10}H_{10}O_5$ is, however, markedly less stable. With bromine water it gives a heavy precipitate.

Like the substance $C_{10}H_{10}O_7$, this compound gives an immediate copious precipitate with 2:4-dinitrophenylhydrazine in 2*N* HCl.

With bleaching powder solution a deep brownish-red colour is obtained, accompanied by formation of a precipitate.

With concentrated H_2SO_4, there is no coloration in the cold, but on warming a pale permanganate-purple colour develops, distinct from the brownish-red given by the substance $C_{10}H_{10}O_7$. The solution in cold H_2SO_4 shows a blue fluorescence in ultra-violet light.

The substance $C_{10}H_8O_4$ is formed, as previously stated, by heating the substance $C_{10}H_{10}O_5$ at 150–170°, and after crystallisation from water, gave the following figures.

Micro-analysis (Schoeller): C, 62·61, 62·81 %; H, 4·29, 4·30 %; OCH_3, nil; $C_{10}H_8O_4$ requires C, 62·50; H, 4·20 %.

0·0530 g. required 2·70 cc. *N*/10 NaOH for neutralisation to phenolphthalein (sharp end-point) corresponding to an equivalent of 196. Theoretical for $C_{10}H_8O_4$ titrating as a monobasic acid, 192. On acidifying the titrated solution, the compound was recovered unchanged.

The substance $C_{10}H_8O_4$ differs from the substance $C_{10}H_{10}O_5$ in its sparing solubility in cold water and chloroform, and can be recrystallised from boiling water. It forms glistening platelets, M.P. 246–250°, with darkening after 230°. It gives a stable deep purple colour with $FeCl_3$ in alcoholic solution and gives no precipitate with 2:4-dinitrophenylhydrazine in 2*N* HCl. With concentrated H_2SO_4 it does not give a colour on gently warming, and with bleaching powder solution it gives a transient orange-red colour. The solution in cold H_2SO_4 shows a blue fluorescence in ultra-violet light.

V. *The substance $C_8H_6O_6$.* This substance was isolated in small yield after a long series of crystallisations from ethyl acetate-light petroleum, carried out on the crude material isolated from the lead precipitate. It crystallises in well-formed hexagonal tablets with slow, and in flint-shaped leaflets with rapid, crystallisation. It melts at 188–190° with effervescence, resets and remelts at 206–210°.

Micro-analysis (Schoeller): C, 48·56, 48·56 %; H, 3·11, 3·18 %; OCH_3, nil; mol. wt., 174, 166. $C_8H_6O_6$ requires C, 48·48 %; H, 3·05 %; mol. wt., 198.

0·1283 g. required 13·68 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 94 (theoretical for $C_8H_6O_6$ titrating as a dibasic acid, 99). The substance was recovered unchanged by acidifying the titrated solution.

It is readily soluble in cold water and in most organic solvents except light petroleum and benzene. With aqueous or alcoholic ferric chloride it gives a stable deep red colour with perhaps a trace of purple. With dilute ferric chloride and dilute alcoholic solutions the colour is brown, but with dilute aqueous solutions it is reddish-purple. It gives no precipitate with bromine water. With bleaching powder solution and a dilute solution of the acid in water, a transient magenta colour is obtained. It does not give a blue fluorescence in H_2SO_4. No precipitate is obtained with 2:4-dinitrophenylhydrazine in 2*N* HCl.

At its first melting-point the substance (per mol.) loses 0·73 mol. H_2O and 0·37 mol. CO_2.

Growth of the organisms on Czapek-Dox solution.

Although in tube experiments, growth of these organisms and the formation of products (as shown by colour reactions) proceeded satisfactorily, greater difficulty was experienced in obtaining the colour reactions on Czapek-Dox solution in flasks than with Raulin-Thom's solution in flasks. As the latter

medium contains, in addition to glucose, considerable amounts of tartaric acid, it was necessary to decide whether the metabolic products arise from tartaric acid, or are true metabolism products of glucose. An experiment on Czapek-Dox solution (glucose being the only source of carbon) was therefore carried out in tubes. 699 tubes, each containing 10 cc. of sterlised Czapek-Dox solution, were sown with a loopful of a spore suspension of *P. brevi-compactum* Dierckx (P 75) and were incubated at 24°. The tubes were periodically tested and after 30 days were taken off. After filtration, 4570 cc. of filtrate, brownish-yellow in colour and showing a green fluorescence, were obtained. The residual glucose by polarimeter was 0·07 %, the bromine absorption 2·20 mg. per cc., the p_H 8·2, and the solution gave a plum colour with ferric chloride. The solution was then evaporated *in vacuo* and worked up as with the routine experiments. The three metabolism products, $C_{17}H_{20}O_6$ (mycophenolic acid), $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$, were shown to be present, and the failure to detect the substances $C_{10}H_{10}O_5$ and $C_8H_6O_6$ was probably due to the smallness of the amount of product. The products $C_{17}H_{20}O_6$, $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$ are therefore certainly, and the products $C_{10}H_{10}O_5$ and $C_8H_6O_6$ are probably, metabolic products of glucose and the presence of tartaric acid is not essential for their formation.

Discussion.

In a series of recent publications [Raistrick *et al.*, 1931] it was repeatedly found in quantitative examination by the carbon balance sheet method of the types of products formed from glucose by large numbers of species and genera, that each group of organisms has its own peculiar biochemical characteristics, while the different species in each group have similar characteristics. The grouping of the species agreed well with the grouping adopted by Thom and Church on morphological grounds.

The present paper may be regarded as the first serious attempt to test the truth of this conclusion in a detailed way, by following various chemical changes during metabolism and by investigation of the chemical nature of a number of the products of metabolism, using as a test series the group of organisms forming the *P. brevi-compactum* series.

The close biochemical relationship of the organisms dealt with has been amply verified. Thus there is a close similarity in the progress of the metabolic processes of the various organisms, *e.g.* in the rate of disappearance of glucose, the increase in bromine absorption of the metabolism fluids, the change in p_H and in the series of colours given by the fluids with ferric chloride after varying periods of incubation. More particularly this relationship is seen in the isolation from almost all the metabolism solutions of a series of products, $C_{17}H_{20}O_6$, $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$, the amounts varying with different organisms.

It is of interest in this connection to note that the total amount of mycophenolic acid isolated seems to vary with the length of time during which

the culture has been kept in artificial cultivation. Thus the largest yields of mycophenolic acid were obtained with the organisms M 3 (1), M 3 (3), M 3 (4) and P 151. Of these, the first three were freshly isolated from spoiled Italian maize, the fourth being received from Prof. Biourge, its history being unknown to us. Alsberg and Black [1913] report that, while a culture of *P. stoloniferum* freshly isolated from Italian maize produced considerable amounts of mycophenolic acid, Dr Thom's type culture of this same organism, which had been kept in artificial cultivation for a considerable time, failed to produce any mycophenolic acid, though in its place was found quite a different substance or mixture of substances, the nature of which was not further investigated. We have had the same experience. Thus our culture, Ad 89, which was obtained through the courtesy of Dr Charles Thom, is the one which was used by Alsberg and Black [1913] for the preparation of mycophenolic acid, and yet in our hands it did not give a trace of mycophenolic acid, but did give the largest yield of the products of lower molecular weight. It seems obvious, therefore, that after almost 20 years in artificial cultivation, this culture had also lost its power of producing mycophenolic acid. Unfortunately, with the remaining cultures, their history is not sufficiently well known to say how long they have been kept in artificial cultivation, although many of them have actually been kept for some time, but the above facts suggest that the power of the organism to produce mycophenolic acid is gradually lost during cultivation over long periods on artificial media.

Moreover, the product mycophenolic acid is not known to be produced by organisms of any other group or series and may therefore be regarded as specifically obtained from organisms of the *P. brevi-compactum* series.

Investigation of the structural relationships of all the various products is now in hand, and it appears probable that their interrelationship will be established and further work may indicate the order of their appearance. In this connection it is interesting to note, from the examination of the series of colours given by the metabolism medium with ferric chloride, that the production of mycophenolic acid takes place almost certainly in the later stages of the metabolic processes.

Summary.

The metabolism of 15 species or strains in the *Penicillium brevi-compactum* Dierckx series has been examined, and mycophenolic acid $C_{17}H_{20}O_6$, together with the following new mould metabolic products $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$ have been isolated. All these products in varying amounts are given by 12 of these species. Two of the three remaining organisms, strains of *P. stoloniferum* Thom and *P. Biourgeianum* Zaleski, although giving no mycophenolic acid, gave in the first case considerable amounts, and in the second case small amounts of the other metabolic products, whilst the third species, *P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski gave none of any of these products.

The best yields of mycophenolic acid are obtained using cultures freshly isolated from natural sources, and such cultures, after keeping over long periods (20 years) in artificial cultivation, tend to lose their power to form mycophenolic acid, but do not lose their power to form the products of smaller molecular weight.

The grouping on morphological grounds of these species in one series is therefore supported by their biochemical characteristics. Thus mycophenolic acid is produced, so far as is known, only by organisms of this series, and is obtained with 12 of the 15 organisms available. Moreover, during the metabolism of these strains, the rate of disappearance of sugar and the changes of p_H, of bromine absorption and of colour reaction with ferric chloride, all show great similarity, the only exception being *P. aurantio-griseum* Dierckx var. *Poznaniensis* Zaleski.

We desire to express our sincere thanks to Mr W. K. Anslow for much technical assistance during the course of this work, and also to Dr M. Pantaleoni of the Health Section. League of Nations, Geneva, for a supply of mouldy Italian maize.

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CLXXII. THE DETERMINATION OF GLUCOSE AND MALTOSE IN AQUEOUS SOLUTION AND IN BROTH AND PEPTONE SOLUTIONS.

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Numerous methods have been worked out by different investigators for the estimation of small amounts of reducing sugars in watery solution and also in biological fluids such as blood and urine. Throughout the work about to be described, a method based on the "cuprous" method of Shaffer and Hartmann [1920] has been used. The method is modified according to the medium in which the sugar is dissolved, and has been extended for use with maltose and with mixtures of maltose and glucose.

Estimation of glucose or maltose in aqueous solution.

The sugar solution is diluted till 0·5–2·0 mg. sugar are contained in 5 cc. 5 cc. quantities are then measured into boiling-tubes and 5 cc. Shaffer-Hartmann reagent added (the composition of the reagent is given on p. 378 of their paper). The tubes are rotated to mix the contents, plugged with cotton-wool and immersed in a bath of briskly boiling water for 40 minutes. After this, they are cooled to room temperature and 10 cc. *N* sulphuric acid are added to each. The contents are stirred and after 2 minutes are titrated with *N*/200 thiosulphate exactly as described by Shaffer and Hartmann.

All estimations are carried out in duplicate and "blanks" are performed on the reagents.

Time of heating. The only significant modification of the Shaffer-Hartmann technique was in the period of heating. It was found that the amount of reduction increased with the time of boiling, rapidly at first and then more slowly. After about 35 minutes the increase is negligible. The chosen period of boiling thus combines a high reduction per mg. of sugar with relative insensitivity to small changes in heating time and hence a uniformity of results. Moreover, maltose reduces rather more slowly than glucose and when estimating mixtures of the two sugars it is desirable to have a standard period of heating which is the same for both.

The results obtained with known solutions of glucose and maltose (B.D.H. A.R. reagents, dried over P_2O_5) are shown in Fig. 1. Both the glucose and the maltose curves are the results of three sets of determinations done at different times and by two different observers. The curve obtained by Shaffer and

Hartmann for glucose is given for comparison. On examination of Fig. 1, it will be seen that our glucose curve has a rather steeper slope than that of Shaffer and Hartmann. This is no doubt owing to the longer period of heating.

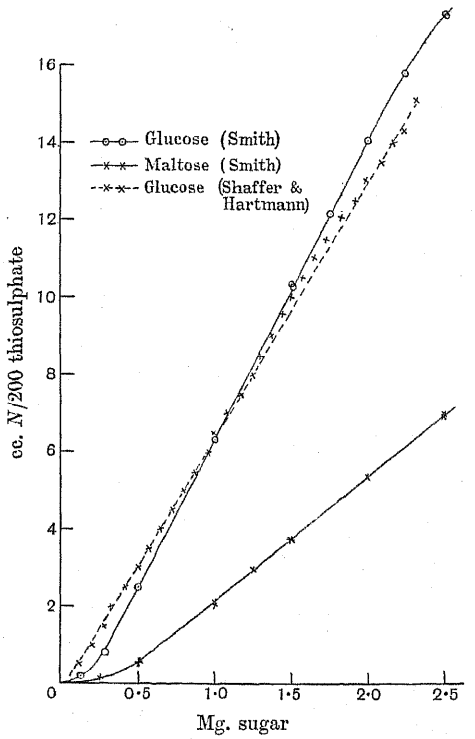


Fig. 1. Titration of glucose and maltose in aqueous solution with thiosulphate.

For glucose the relationship between the amount of thiosulphate used up and the amount of sugar present is a linear one within the range 0·3–2·0 mg. glucose. For maltose the relationship is linear within the range of 0·5–2·5 mg. Amounts of maltose greater than 2·5 mg. were not investigated. With very small amounts of sugar, the relationship is no longer linear; presumably some sugar is destroyed by the heat before it can reduce the copper. With the longer period of boiling the amount destroyed is somewhat increased. It is probably not feasible to estimate amounts of sugar less than 0·3 mg. with great accuracy by this method.

Estimation of mixtures of maltose and glucose.

If conditions can be found under which maltose will hydrolyse quantitatively to glucose without producing other reducing by-products, then the analysis of mixtures of maltose and glucose can be carried out by the Shaffer-Hartmann method.

Maltose does not hydrolyse with such readiness as lactose or sucrose. It

was found that in order to obtain complete hydrolysis, the solution must be at least 1·6 *N* acid and the heating time must be at least 35 minutes.

The procedure adopted is to measure 5 cc. of the solution to be hydrolysed into a boiling-tube and to add 1 cc. of 10*N* sulphuric acid. The tubes are plugged and immersed in a bath of boiling water for 40 minutes. After this time they are cooled and one drop of cresol red is added to each. The solutions are now neutralised by the addition of 40 % sodium hydroxide drop by drop, and then just acidified by the addition of *N* sulphuric acid (2 or 3 drops usually suffice). Now 5 cc. Shaffer-Hartmann reagent are added and reduction and titration are carried out as usual. The end-point is slightly more difficult to read in the presence of the indicator but presents no serious difficulty once the observer is familiar with the colour change. Simultaneous estimations are carried out on the sugar solutions without hydrolysis.

The additions of sulphuric acid and sodium hydroxide do not affect the blank value so that one determination of the reagent blank suffices for the hydrolysed and unhydrolysed portions.

This method was tested by estimating a solution of maltose before and after hydrolysis.

Before hydrolysis, maltose recovered = 1·62 mg.
After hydrolysis, glucose recovered = 1·65 mg.

Calculation of the amounts of glucose and maltose in mixtures.

As has been stated above, with both maltose and glucose a certain amount of sugar is destroyed without causing reduction of the copper. In mixtures, the amount of each sugar destroyed is proportional to its concentration in the mixture and the total amount destroyed is 0·23 mg. under the specified conditions.

A computation of the actual amounts of glucose and maltose present is somewhat complex, but to a first approximation the values can be calculated as follows:

Let the number of mg. of maltose present be X.
,, ,, glucose ,, Y.

Let t_1 cc. $N/200$ thiosulphate be used in titrating before hydrolysis and let t_2 cc. $N/200$ thiosulphate be used in titrating after hydrolysis.

Now over the straight portions the slope of the glucose curve corresponds to 8·00 cc. $N/200$ thiosulphate per mg. glucose, and the corresponding figure for the maltose curve is 3·15 cc. per mg. If the straight lines are produced they cut the base line at a point corresponding to 0·23 mg. sugar approximately. If the values of sugar recovered are taken not from the origin but from the point where the lines cut the base line, two simultaneous equations can be obtained. Let these new values be X' and Y' respectively.

After hydrolysis all the sugar $(X' + Y')$ is present as glucose, therefore

$$t_2 = 8\,(X' + Y') \qquad (1).$$

Before hydrolysis X' is present as maltose, Y' as glucose, therefore

$$t_1 = 3{\cdot}15\,X' + 8Y' \qquad (2).$$

t_1 and t_2 are determined experimentally and hence X' and Y' can be found. Now the true values for X and Y can be calculated to a first approximation thus:

$$X = X' + \frac{0{\cdot}23 \times X'}{X' + Y'}$$

and

$$Y = Y' + \frac{0{\cdot}23 \times Y'}{X' + Y'}.$$

In practice the calculation is not nearly so tedious as it appears to be on paper.

That the formula represents the facts with reasonable accuracy may be seen by the following estimations of a known mixture of maltose and glucose.

Sugar added		Sugar recovered	
Maltose %	Glucose %	Maltose %	Glucose %
0·300	0·15	0·311	0·151

Estimation of glucose and maltose in peptone water or tryptic digest medium.

Separation of the sugar from other reducing substances. Bacteriological culture media made from muscle digest or peptones present peculiar difficulties when it is desired to estimate the reducing sugar contained in them. For such media contain a large proportion of non-sugar reducing substances which are not completely removed by the ordinary methods of protein precipitation.

Precipitation with phosphotungstic acid removes a larger proportion of these substances than tungstic acid or any of the recognised mercury reagents, but even so the filtrate after precipitation contains 15–20 % of its original nitrogen and has a moderate reducing power. Animal charcoal and Lloyd's reagent do not remove these reducing substances.

Kerly [1931] gives a method for the estimation of glucose in muscle, in which the proteins are first removed and the glucose in the filtrate is then precipitated with copper hydroxide and lime by the method of Van Slyke [1917]. Using the technique described by Kerly, we have repeated this process for two broths A and B containing glucose, with simultaneous estimation of the total nitrogen content at the various stages. The results, corrected for the dilution involved, are as follows:

	Percentage original nitrogen remaining	
	A	B
Original broth	(100)	(100)
Phosphotungstic acid filtrate	15·2	20
Filtrate after 1st copper-lime precipitation	4·6	3·5
Filtrate after 2nd „ „	2·3	2·1

The final filtrate is shown to be relatively free from nitrogenous material, the actual nitrogen content being 0·01–0·014 mg. N per cc. The medium used was a tryptic digest of horse muscle, which is very rich in nitrogenous material. With peptones the percentage nitrogen remaining after the above process would probably be less.

Estimation of the separated sugar. Kerly re-dissolved her "copper-lime" precipitate in acid, boiled it with alkaline tartrate and used the reduced copper to reduce a complex phosphomolybdate mixture with the production of an intense blue colour. This in its turn was converted into a colourless compound by titration with standardised permanganate.

This method was tried by us but found to be inaccurate for the following reasons.

(1) The instability of the blue compound which necessitates very rapid work.

(2) The difficulty of reading the end-point accurately when working rapidly.

(3) The insensitivity of the method.

Attempts to measure the blue colour by means of a photoelectric colorimeter also failed owing to the rapid and eventually complete fading of the colour even when kept in stoppered bottles in the dark.

Shaffer-Hartmann method. Since the Van Slyke method for precipitating sugars involves the addition of copper sulphate, it seemed desirable to use one of the alkaline copper tartrate methods for estimating the sugar. The Shaffer-Hartmann small-scale method was therefore tried. These workers point out the necessity for keeping the concentration of Cu^{++} as low as possible in order to avoid a spontaneous back reaction with conversion of some of the excess cupric to cuprous salts. In Kerly's technique, the amount of cupric ions present in the final filtrate is four times as great as that recommended by Shaffer and Hartmann. We found that the concentration of cupric salt recommended by Kerly can be halved without deleterious results but that further reduction in the amount causes incomplete precipitation of the sugar.

When half Kerly's concentration of copper is used, the concentrations of the tartrate, oxalate and carbonate in the Shaffer-Hartmann reagent must be doubled to allow for the extra concentration of copper. This doubling of the concentration of the reagent proved to have no effect on the amount of reduction caused by the glucose.

The procedure for estimating glucose or maltose in peptone or broth is thus as follows.

Special reagents.

Phosphotungstic acid. 8 % in 1·7 *N* HCl. The HCl solution is better for this purpose than that in H_2SO_4 as with the latter solution calcium sulphate tends to precipitate.

Alkaline tartrate reagent.

	%
Anhydrous sodium carbonate	8·0
Tartaric acid	1·5
Potassium oxalate ($1H_2O$)	3·68
Potassium iodide	1·0
Potassium iodate	0·07

Milk of lime 5 %. 110 g. calcium chloride are dissolved in 500 cc. distilled water and 100 cc. 40 % NaOH added slowly with stirring. The precipitate is separated on the centrifuge, washed twice with 1 litre of water and finally suspended in water to make a volume of 1 litre.

Method.

10 cc. of the broth containing sugar are measured into a boiling-tube and 19·5 cc. phosphotungstic acid and 0·5 cc. 10*N* HCl added. The contents are stirred, left 2 minutes and filtered through dry paper. 15 cc. of the filtrate are measured into a 50 cc. volumetric flask, and 5 cc. 10 % $CuSO_4$ are added. 40 % NaOH is added, drop by drop, until the copper just begins to precipitate, then 10 cc. milk of lime are added and the contents made up to the mark. The mixture is left for half an hour, then 40 cc. are measured into a centrifuge-pot and spun for 3 minutes. The precipitate is re-dissolved by the addition of 20 cc. *N* HCl and the volume made up to 40 cc. 20 cc. are transferred to a centrifuge-pot and 20 cc. of 0·5 *N* NaOH added. The contents are stirred, left for half an hour and spun. The precipitate is re-dissolved in 5 cc. *N* HCl and the volume made up to 20 cc. During the above process, the solutions can safely be left in the acid state but should not be allowed to stand longer than the specified time in the alkaline state, and the precipitate should be dissolved immediately after spinning.

For the reduction, 5 cc. of the final solution are measured into each of two boiling-tubes and into two more tubes, which are used as blanks, 0·5 cc. 10 % $CuSO_4$ and 5 cc. water are measured. To all tubes 5 cc. alkaline tartrate are added and the tubes are plugged and plunged into a bath of boiling water for 40 minutes. They are then cooled, acidified and titrated exactly as for aqueous solutions. To be on the safe side, a small quantity of solid potassium oxalate is added immediately before titration as insufficient oxalate prevents a clear end-point being obtained, and in these cases the calcium present combines with some of the oxalate.

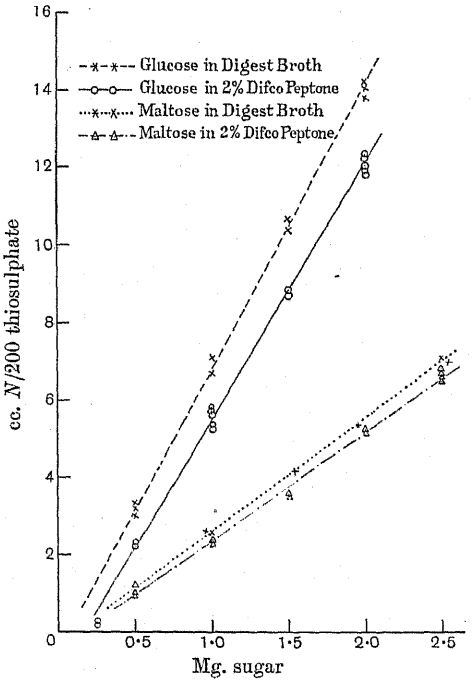


Fig. 2. Titration of glucose and maltose from broth and peptone solution with thiosulphate.

The method was tested for 2 % Difco proteose peptone and also for tryptic digest broth by determining the reducing value of the medium with and without the addition of known amounts of glucose and maltose, and constructing graphs as for glucose in water. The reducing value when there is no added

sugar must be subtracted from the values when sugar is added to obtain comparable graphs.

These graphs are shown in Fig. 2.

Each graph has been constructed from three separate sets of readings, the result of observations by two different experimenters, and in the case of broths on different batches of broth. Though the duplicates do not agree quite so closely as in the glucose-water estimations, there is never any substantial difference between the three sets of results.

It will be seen that glucose and maltose in digest broth and maltose in Difco peptone give practically the same reducing value per mg. sugar as the same sugars in aqueous solution, *i.e.* there appears to be no loss of sugar during precipitation of bases, *etc.*

When glucose in Difco peptone is used, however, the recovery is only about 85 %. Apparently in this case some sugar is lost either in the phosphotungstic acid precipitate or elsewhere. Since the graph has been reproduced from 3 sets of observations, there seems no reason for doubting that this is a true result. It follows that in estimating glucose in Difco peptone solution care must be taken to read from the glucose-peptone curve in Fig. 2. It has not been ascertained whether the low results given by 2 % Difco proteose peptone containing glucose would also be obtained if other peptones were substituted.

Fermentation of the medium.

For peptone solution, the reducing value in the absence of added sugar is very small (0·47 cc. $N/200$ thiosulphate).

For peptone-meat extract or tryptic digest broth, this blank value is much higher. For the maker of culture media, it is of interest to ascertain what portion of this blank value is due to sugar present in the finished medium and what portion is due to non-sugar reducing substances.

This can be ascertained by estimating the reducing value before and after fermentation with yeast.

A sample of baker's yeast is washed three times with a large volume of distilled water to remove any adhering sugar and then pressed. The cake can be preserved moist at 4° for upwards of 14 days without losing its activity. 0·2 % yeast is added to one sample of the medium and incubated at 35–37° for 5 hours (2½ hours proved insufficient). After this period, the reducing value of the unfermented and fermented samples is determined and the difference gives the sugar content of the original broth.

The following results may be of interest:

Type of broth		Glucose equivalent: Before fermenting %	Glucose equivalent: After fermenting %	Glucose in broth %
Tryptic digest of horse muscle	1	0·031	0·028	0·003
	2	0·044	0·016	0·028
	3	0·065	0·028	0·037
2 % Difco veal extract	1	0·034	0·018	0·016
	2	0·108	0·034	0·074

It will be noted that the sugar content of the broth is quoted as glucose. As a matter of fact, no experiment was made to determine the nature of the reducing sugar present.

Mixtures of maltose and glucose in peptone and broth.

If the culture medium contains a mixture of maltose and glucose the hydrolysis is carried out immediately after precipitation with phosphotungstic acid.

15 cc. of the acid filtrate are measured into a boiling-tube and 1 cc. concentrated HCl added. The tubes are plugged and immersed in a bath of boiling water for 1 hour. (The longer period of hydrolysis is made necessary by the larger volume of liquid being used.) After hydrolysis, the contents of the tube are transferred to a 50 cc. volumetric flask, the tubes are rinsed with water, the rinsings added to the main liquid and 5 cc. of 10 % copper sulphate are added. 15 cc. of the unhydrolysed phosphotungstic acid filtrate are measured into another 50 cc. flask and the contents of both are put through two "copper-lime" precipitations. Reduction and titration are carried out as usual.

Maltose in broth or 2 % Difco peptone can be recovered quantitatively as glucose after hydrolysis in this manner.

Summary.

1. The method of Shaffer and Hartmann for the determination of small amounts of glucose has been extended to the determination of maltose and of mixtures of maltose and glucose.

2. The method has been modified for the determination of maltose and glucose in peptone and digest culture media.

3. In such media, 0·1 %–0·4 % glucose or maltose can be recovered with an error not exceeding 5 % of the amount estimated.

The author wishes to express her thanks to Dr J. W. Trevan and to Mr C. G. Pope for interest and advice throughout the work and to Miss C. Gross who carried out duplicate determinations on all the curves.

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CLXXIII. THE EFFECT OF HEAT ON SUGAR SOLUTIONS USED FOR CULTURE MEDIA.

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It is a well-established fact that some types of culture medium are rendered unsuitable for the growth of certain bacteria and fungi if sterilised by autoclaving, and in the literature repeated references to this phenomenon are found. But so far as the author is aware little work has been carried out to determine the nature of the change caused by the heating of the medium.

Pope (private communication) found that cultures of *C. diphtheriae* grown on medium containing Difco proteose peptone, glucose and maltose did not produce good toxin if the medium had been autoclaved. Moreover he found that the heat-labile factor in the medium lay not in the peptone but in the sugar-inorganic salt solution in which the peptone was dissolved.

This suggested that it would be interesting to determine the stability of glucose-maltose-salt mixtures towards heat, under conditions similar to those commonly used in the preparation of culture media.

That sugars are readily decomposed by strong alkalis such as caustic soda is a very well-known fact, the rate of decomposition increasing with the concentration of alkali used. The oxidation of glucose by hydrogen peroxide has been studied by Löb [1911] and by Witzemann [1920] and the oxidation by iodine has been investigated by Kappanna [1928]. All three workers found that phosphates increase the rate of oxidation of the sugar. Kappanna and Löb find the rate of oxidation to be increased by raising the p_H value and Kappanna maintains that the only rôle of the phosphate is in buffering the solution in the neighbourhood of p_H 8 and preventing the fall of p_H due to acid produced from the glucose. Löb, on the other hand, states that when hydrogen peroxide is the oxidisng agent, phosphate exerts a specific accelerating effect when the p_H value is kept constant and Witzemann does not think reaction to be of primary importance—the main factor influencing the rate of oxidation being phosphate concentration.

The following experiments were carried out with a view to determining whether heating in the autoclave caused destruction of maltose and glucose and if so whether phosphates played an important part in the change. It was also considered to be of interest to investigate the relative effects of heating sugar solutions at 100° and under excess pressure in the autoclave.

Methods.

The sugars were estimated by the Shaffer-Hartmann method [1920] modified as described in the previous paper. For maltose and glucose-maltose mixtures the estimation was carried out before and after hydrolysis, and the amounts of each sugar calculated. There is the possibility that some other reducing substance may be produced in the solutions during autoclaving. The presence of such a substance would introduce an error into the calculations. Harden [1911], in discussing the degradation products of glucose, mentions many which are not fermentable by yeast but none which is to any marked extent fermented. Although there is still the possibility that fermentable reducing substances other than sugars do exist, we have been able to find no evidence of this in the literature. It was therefore considered to be of interest to determine the effect of yeast fermentation on the autoclaved solutions and it was found that after such fermentation the reducing power fell to less than 4 % of its value before fermentation. So the solutions contain little or no non-fermentable reducing substances and we have assumed that glucose and maltose are the only reducing substances present. Our calculations are based on this assumption.

Exp. 1. Effect of autoclaving glucose-salt solutions.

The medium was composed as follows:

Glucose	1·5 g.
K_2HPO_4	2·0 g.
Na_2HPO_4	2·0 g.
$MgSO_4, 7H_2O$	0·2 g.
$CaCl_2$	0·1 g.
Glacial acetic acid	10·0 cc.
Cresol red	0·02 g.
NaOH to p_H 8·0	
Distilled water to 500 cc.	

The chemicals used were B.D.H. A.R. chemicals and weighings were accurate to 0·5 %. A portion was reserved as a control, then 100 cc. quantities were placed in bottles and autoclaved at 15 lbs. pressure for various times. The temperature was raised and lowered as rapidly as possible to obviate excessive heating and the period of heating quoted is the actual time when the pressure was 15 lbs. The result is shown in Table I.

Table I. *Showing effect of autoclaving on glucose solutions.*

Time of autoclaving	% glucose present	% of original glucose remaining	p_H
Control: not heated	0·280	100	7·90
5 minutes	0·252	90	7·76
10 ,,	0·236	84	7·64
20 ,,	0·216	77	7·54
40 ,,	0·200	71·5	7·41

Table I shows that in the presence of 0·8 % phosphate, glucose is gradually destroyed with simultaneous lowering of the p_H value.

Exp. 2. Effect of autoclaving maltose solutions.

Exp. 1 was repeated exactly except that 0·6 % maltose was substituted for the glucose.

The results are shown in Table II.

Table II. *Showing the effect of autoclaving maltose solutions.*

Time of autoclaving	% maltose present	% glucose present	Total	% original maltose	% total sugar remaining	p_H
Control: not heated	0·581	0·009	0·590	(100)	(100)	7·96
5 minutes	0·397	0·107	0·504	68·5	85·4	7·46
10 „	0·298	0·188	0·486	51·3	82·5	7·37
20 „	0·246	0·196	0·442	42·4	75·0	7·16
40 „	0·218	0·192	0·400	37·6	68·0	7·04

Table II shows that when autoclaved in the presence of phosphates, the solution becomes gradually poorer in maltose and richer in glucose; in other words hydrolysis of maltose to glucose occurs. The glucose in its turn is slowly destroyed as is shown by the gradual fall in total sugar content. Probably it is oxidised to an acid for the hydrion concentration rises as autoclaving proceeds.

Exp. 3. Effect of autoclaving mixtures of maltose and glucose.

The composition of the medium was the same as for Exps. 1 and 2, except that it contained both 0·6 % maltose and 0·3 % glucose. Portions were autoclaved at 15 lbs. pressure for various times, after which samples were taken, diluted suitably and their sugar contents estimated. The results are shown in Table III.

Table III. *Showing the effect of autoclaving a glucose-maltose solution containing 0·8 % phosphate.*

Time of autoclaving	% maltose present	% glucose present	Total	p_H
Control: not heated	0·619	0·267	0·886	7·68
5 minutes	0·399	0·368	0·767	7·24
10 „	0·345	0·384	0·728	7·19
20 „	0·342	0·392	0·734	7·08
40 „	0·200	0·440	0·640	6·84

Exp. 4.

In view of the work of Löb, Witzemann and Kappanna quoted above it was considered of interest to repeat the experiment on the heating of maltose-glucose mixtures, using different concentrations of phosphates.

Three batches of medium were made up, the composition being as follows:

	A (%)	B (%)	C (%)
Glucose	0·15	0·15	0·15
Maltose	0·3	0·3	0·3
Cresol red	0·002	0·002	0·002
Na_2HPO_4	0·00	0·10	0·20
K_2HPO_4	0·00	0·10	0·20

All three batches were adjusted with NaOH to p_H 8·0 before autoclaving at 15 lbs. pressure.

The results are shown in Table IV.

Table IV. *Showing influence of phosphates on the heat change in sugars.*

Period of autoclaving	No phosphate A Maltose %	Glucose %	Total %	p_H	0·2 % phosphate B Maltose %	Glucose %	Total %	p_H	0·4 % phosphate C Maltose %	Glucose %	Total %	p_H
Control: not heated	0·311	0·151	0·462	7·55	0·319	0·141	0·460	7·94	0·313	0·148	0·461	7·82
5 minutes	0·271	0·168	0·439	7·43	0·227	0·181	0·408	7·44	0·213	0·201	0·414	7·66
10 „	0·225	0·201	0·426	7·15	0·147	0·224	0·371	7·17	0·111	0·223	0·334	7·53
20 „	0·253	0·181	0·434	7·22	0·165	0·224	0·389	7·12	0·127	0·228	0·355	7·48

Table IV shows that the hydrolysis of maltose to glucose and the destruction of glucose caused by heating are both accelerated by increasing the concentration of phosphates.

Many workers who realise that autoclaving causes deleterious effects in their culture media, have substituted a period of heating at 100° on three successive days.

In order to determine whether or not sugar solutions could be heated at the lower temperature for long periods without causing changes in the sugar content, the following experiment was devised.

Exp. 5. Effect of boiling mixtures of maltose and glucose.

The medium was composed as follows:

Maltose	1·5 g.
Glucose	0·75 g.
K_2HPO_4	1·0 g.
Na_2HPO_4	1·0 g.
Cresol red	0·02 g.
NaOH to p_H 8·0	
Distilled water to 500 cc.	

The mixture was placed in a flask fitted with an efficient reflux condenser to prevent concentration during boiling and during the whole period of boiling

Table V. *Showing the effect of boiling maltose-glucose mixtures.*

Period of boiling	% maltose present	% glucose	Total
Control: not heated	0·261	0·157	0·418
0·5 hours	0·179	0·196	0·375
1·0 „	0·120	0·215	0·335
2·0 „	0·089	0·217	0·306
4·0 „	0·043	0·217	0·260
6·0 „	Not measurable	0·243	0·243
7·0 „	„	0·246	0·246

a stream of compressed air was bubbled through the solution. Portions were withdrawn after varying periods and analysed for maltose and glucose.

The results are shown in Table V.

The above experiment was repeated, bubbling nitrogen through the liquid instead of compressed air. The results were, within the limits of the experiment, identical with those shown in Table V.

Discussion.

It is shown in Exp. 1, that glucose is slowly destroyed by autoclaving in the presence of phosphates. With 0·8 % phosphate and 40 minutes' autoclaving at 15 lbs. pressure, the concentration of glucose is reduced to about 70 %. Exp. 2 shows that maltose disappears from the solution more rapidly than glucose, since after 40 minutes only 38 % remains. If the change in maltose concentration is entirely due to hydrolysis to glucose, then the loss in total sugar from the solution will be caused solely by the destruction of glucose. The fact that in Exps. 2 and 3 the loss in total sugar during 40 minutes' autoclaving is respectively 32 % and 28 % compared with a loss of 28·5 % of glucose in Exp. 1 for the same heating period, is strong evidence that destruction of glucose accounts for the whole of the loss of sugar from glucose-maltose mixtures.

Exp. 4 clearly shows that the changes caused by heat are accelerated by phosphates. It will be seen, however, that in all experiments the reaction of the solutions becomes less alkaline as autoclaving proceeds. Moreover, the fall in p_H value is greater in the absence of phosphates and becomes progressively less as their concentration increases.

It is possible therefore that the action of phosphates is merely one of buffering the solution at a more alkaline reaction, for most workers are agreed that destruction of glucose is accelerated by increase in hydroxyl ion concentration. The author is of opinion, however, that the change produced by lowering the phosphate concentration is too great to be accounted for by the relatively small lowering of the hydroxyl ion concentration which results. In this connection the observation of Pope (private communication) is of interest. He noted that the deleterious effect caused by heat in medium prepared from Difco peptone and sugars for the production of diphtheria toxin was markedly greater in the presence of phosphates.

When the phosphate concentration was reduced to 0·2 %, toxin production was almost as good in autoclaved medium as in medium sterilised without heat. In medium such as Pope used, any fall in p_H value caused by acid production should be as adequately checked by the peptone as by the phosphates. The effect of phosphate is probably therefore not simply one of maintaining the alkalinity, but is more probably a specific accelerating effect on the destruction of sugars.

Exp. 5 shows the effect of boiling maltose-glucose solutions in air. To compare the effect with that of autoclaving the same solutions, Exp. 5 must

be compared with Exp. 4 B which is its proper control. It will be seen from Exp. 4 B that autoclaving for 20 minutes reduces the concentration of maltose to 52 % and that of the total sugar to 84 % of the amounts originally present. In Exp. 5 after one hour's boiling in air, the concentration of maltose was 46 % of the original amount and that of total sugar 81 %. It therefore appears that as regards the power of destruction of maltose and glucose, one hour at 100° is approximately equivalent to 20 minutes under 15 lbs. excess pressure. If, therefore, the medium to be sterilised contains maltose or glucose it does not appear that steaming for 20 minutes on three successive days would offer any advantage over autoclaving.

Summary.

1. Autoclaving at 15 lbs. pressure causes hydrolysis of maltose to glucose and slow destruction of the glucose with the production of acid.

2. The changes in sugar concentration on autoclaving are accelerated by the addition of phosphates.

3. As regards the power of destruction of maltose and glucose, one hour at 100° is approximately equivalent to 20 minutes under 15 lbs. excess pressure.

The author wishes to express her thanks to Mr C. G. Pope and Dr J. W. Trevan for the interest they have taken in this research.

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CLXXIV. NOTE ON THE NITROGEN CONTENT OF CERTAIN PROTEINS.

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In the course of their investigations, many workers require to estimate small amounts of protein material; several methods are available for the determination of protein, but for small amounts perhaps the best is the determination of total nitrogen content, as on the micro-scale this is rapid, accurate and economical of material. But in order that this method shall give a true estimation of the amount of protein present, it is essential that the nitrogen content of the protein in question be accurately known.

In the standard textbooks, proteins are variously quoted as containing 15–16 % nitrogen, the generally accepted factor for the conversion of nitrogen to protein being 6·25. Hausmann [1899] found the nitrogen content of crystalline egg-albumin to be 15·51 %, that of crystalline serum-albumin to be 14·60 % and of horse serum-globulin to be 16·17 %. Osborne [1903] quotes the nitrogen content of caseinogen as 15·62 % and of egg-albumin as 15·51 %. He investigated the nitrogen contents of various vegetable proteins which he found to be higher than those of proteins of animal origin. His values [1924] for different vegetable proteins varied from 18·84 to 15·62 % with an average of about 17·3 %. Miller and Chibnall [1932] found a nitrogen content for various grass-proteins varying from 12·3 to 14·0 % of dry weight but their preparations admittedly contained non-nitrogenous impurities. Banzhaf [1914] estimated the nitrogen contents of various immune sera, some of which were in the crude state and some purified by ammonium sulphate precipitation. The values found for nitrogen were much lower than the earlier figures quoted above, varying from 14·07 % for purified antidiphtheria pseudoglobulin to 13·09 % for crude antitetanus serum. Felton [1932] on the other hand has found higher values for Felton protein, varying from 14·9 to 15·7 %.

In view of the large variation in results quoted above and also of the fact that 6·25 is still accepted by many workers as a universal protein-nitrogen factor regardless of the nature of the protein, we have thought it desirable to carry out nitrogen determinations on known weights of certain proteins encountered during investigations on toxins and sera.

Purification of the protein.

The method of purifying the protein varied according to the nature of the material.

Diphtheria toxin and toxoid. The culture filtrate was acidified with hydrochloric acid to p_H 4·6–4·7 (for toxin) or to p_H 4·2–4·3 (for toxoid). The mixture was left until a floccular precipitate began to form, and this was separated by means of a centrifuge, washed first with buffer at p_H 4·0 and finally with distilled water. It was then emulsified in water and taken into solution by the addition of the minimum quantity of sodium hydroxide, filtered to remove any extraneous solid matter and re-acidified. The precipitate was separated and washed as before and finally dried to constant weight over phosphorus pentoxide. The material thus purified contained 35–106 flocculation units per mg. of dry weight. For nitrogen determinations a known weight was dissolved in 0·7 % saline in quantity sufficient to bring the nitrogen content to 0·3–0·8 mg. per cc.

Diphtheria antitoxin and normal pseudoglobulin. The serum was fractionated with ammonium sulphate and dialysed. The pseudoglobulin was electrodialysed to remove any remaining euglobulin, which was filtered off. The clear filtrate was dried over phosphorus pentoxide to constant weight.

Serum-albumin. The globulins from the serum were removed by precipitation with ammonium sulphate and the filtrate was dialysed till free from ammonium sulphate and dried to constant weight over phosphorus pentoxide.

Purified type I pneumococcus antibody. The Felton protein was obtained, after a preliminary salting out of the globulins from the serum with ammonium sulphate, by dialysis of this fraction against tap and distilled water, and an isoelectric fractionation of the resulting mixture to remove euglobulin and pseudoglobulin. The euglobulin was precipitated at p_H 5·0 and separated from the liquid by centrifuging; the Felton protein was precipitated from the supernatant liquid containing pseudoglobulin and Felton antibody, at p_H 6·8. It was collected by means of a centrifuge and dissolved in 3 % saline. When required for the nitrogen determination the protein was dialysed from salt against distilled water, separated by means of a centrifuge and washed several times with distilled water. It was dried over phosphorus pentoxide to constant weight.

Method of nitrogen determinations.

Nitrogen determinations were carried out by a modification of Pregl's method [1924], a small scale method based on the classical one of Kjeldahl. Four to seven estimations were carried out on each protein sample, the whole process taking about an hour and a half. By this means we have been able to determine quantities of nitrogen of the order of 0·1 mg. with a standard deviation of $\pm$ 0·002 mg. In certain cases the standard deviation for a series of four determinations has been as low as $\pm$ 0·0005 mg. In fact, in the results quoted in Table I the error in weighing sometimes approximates to the error

in nitrogen determinations and the standard deviations quoted were computed by taking both errors into account. It is known that the usual Kjeldahl method of nitrogen estimation does not recover all the nitrogen from certain materials, and that to obtain full recovery the Arnold-Gunning modification must be used. In order to determine whether this was necessary for the materials in question, a number of estimations were made by our usual modification in parallel with estimations using the Arnold-Gunning modification. There was no difference in the amount of nitrogen found with the two methods.

Table I. *Nitrogen content of certain proteins.*

No.	Type of protein	Nitrogen %	Nitrogen factor
TMR 597	Diphtheria toxoid*	14·07 ±0·06	7·12 ±0·03
TMJ 822	,,	14·64 ±0·04	6·82 ±0·02
TV 658	Diphtheria toxin	14·27 ±0·13	7·01 ±0·06
TX 659	,,	14·43 ±0·10	6·92 ±0·05
SS 42	,,	13·62 ±0·23	7·32 ±0·11
TP 592	,,	14·05 ±0·22	7·11 ±0·11
TR 642	,,	14·26 ±0·07	7·01 ±0·03
TJ 652	,,	14·60 ±0·08	6·78 ±0·04
PC 1667	Purified antidiphtheria pseudoglobulin	13·74 ±0·21	7·35 ±0·10
PC 1744	,, ,,	13·57 ±0·04	7·37 ±0·02
PC 1760	,, ,,	14·53 ±0·31	6·88 ±0·15
PC 1749	,, ,,	14·93 ±0·08	6·69 ±0·04
RX 2008	,, ,,	14·16 ±0·18	7·05 ±0·09
	Normal pseudoglobulin	14·12 ±0·17	7·08 ±0·08
	Normal serum-albumin	13·82 ±0·50	7·23 ±0·25
NC 3	Purified type I pneumococcus antibody	14·10 ±0·07	7·09 ±0·04
	Mean of whole series	14·18	7·05

* The diphtheria toxins were prepared from cultures on the different types of culture medium. No significant difference in nitrogen content was found when different types of medium were used.

Discussion.

Of these 16 estimations on different proteins only one (PC 1744) gives a nitrogen factor definitely greater than 7·25 and only one (PC 1749) a factor definitely less than 6·80. The mean factor for the whole series is 7·05, corresponding to 14·18 % nitrogen. The divergence between the upper and lower factors given is only slightly greater than our usual error of nitrogen determination. This is a markedly lower nitrogen content than the older results quoted by Osborne or Hausmann but is in excess of most of the results quoted by Banzhaf for immune sera or by Miller and Chibnall for grass-proteins. A nitrogen content might appear to be greater than the true value if the material were contaminated with impurities richer in nitrogen than the true protein, such as certain amino-acids, amides, ammonium sulphate, *etc.* High nitrogen values would therefore result if precautions were not taken to separate the proteins from such impurities. The values obtained would on the other hand be too low if the protein were contaminated with non-nitrogenous impurities such as carbohydrates, salts, dust or silica. They would also be low if the nitrogen were not recovered quantitatively by the Kjeldahl method.

In order therefore to assess the reliability of the results obtained it is necessary to examine very carefully the methods used in the preparation and analysis of the protein material.

Many proteins are prepared by fractionation of the original material with ammonium sulphate. If this salt is not removed completely by subsequent dialysis, error will be introduced. In the preparation of the pseudoglobulin, albumin and Felton protein used by us, ammonium sulphate was used but great care was taken to ensure its complete removal by dialysis. The fact that no significant difference in nitrogen content was obtained for serum-albumin, Felton protein, antidiphtheria and normal pseudoglobulin and diphtheria toxin, proteins derived from very different sources and purified by different methods, makes it improbable that there is any appreciable error in the results due to contamination of the purified product with impurities, nitrogenous or otherwise. As regards the recovery of nitrogen by the modified Kjeldahl method employed we have tested the recovery of nitrogen from solutions of ammonium sulphate, urea and asparagine. The results are usually about 0·4–0·5 % below the theoretical so that it is probable that the percentages of nitrogen to dry weights quoted in Table I ought to be raised by 0·4–0·5 %.

It thus appears improbable that the values quoted in the table differ from the true values for the types of protein investigated by amounts greater than the deviations given.

Summary.

The figures for nitrogen content of various proteins usually quoted vary from 15 to 16 %, or even higher. The nitrogen content of certain proteins derived from diphtheria toxin, and from diphtheria and other sera, when determined by the micro-Kjeldahl method is considerably lower, varying from 13·57 to 14·64 %. The average is 14·18, giving a nitrogen factor of about 7·05.

We wish to express our thanks to Mr C. G. Pope for his interest and advice.

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CLXXV. PROTEOLYSIS IN TUMOURS.

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Introduction.

Among the most difficult questions regarding the metabolism of tumour cells are those of the content of proteolytic enzymes in the cell itself and the linkage between the proteolytic functions and other metabolic processes as well as their relations with malignancy.

It has been admitted for a long time [Neuberg and Gottschalk, 1925] that tumour tissue has a high content of so-called autolytic enzymes and also a certain degree of proteolytic activity against proteins of normal tissues (heterolytic function); and this ought to be an explanation for the liquefying and destroying action, which tumour growth exerts on surrounding tissues. There are also some earlier observations of Abderhalden and co-workers [1909; 1910] on atypical peptolytic activities of tumour tissues against some artificial polypeptides.

During the last few years we find the work of Waldschmidt-Leitz and Schäffner [1930], who, applying to the study of tumour enzymes the methods and conceptions of the Willstätter school, found that the cathepsin was abundant in a rat sarcoma and showed during tumour growth an increasing degree of self-activation, *i.e.* it required less and less activation by H_2S for action on gelatin. As activation by H_2S and sulphydryl bodies (SH-glutathione, cysteine, *etc.*) characterises the catheptic enzymes of cells according to the ideas of these authors, their finding would signify that the tumour as it grows contains more and more activators, represented by reducing substances like the foregoing. A relation of these substances and therefore of the increased proteolysis with the lowered oxidation in tumours is consequently supposed.

Krebs on the contrary in an extensive study of proteolytic enzymes of normal and tumour cells could find no special features in enzymes from tumour tissues of man and animals. He tried to demonstrate [1930] that the promoting action of substances containing the —SH group and of HCN on cathepsins was not a true activation but rather the effect of a combination with traces of inhibiting, enzyme-poisoning heavy metals ("*Komplexbildung*"). He then [1931] stated that tumours are to be ranged as to proteolytic activity below some normal organs (kidney in rat, thyroid gland in man), if the cellular enzymes are studied by appropriate methods, *e.g.* if care is taken to enhance

to a maximum the enzymic function by adding to the mixture of tissue extract *plus* substrate (gelatin) a proper concentration of cysteine, which is able to combine with heavy metals, preventing in this way any disturbance of enzymic function due to traces of such metals.

Stern [1931] studied the action of cell enzymes on the proteins of the extract themselves, *i.e.* he developed a method corresponding to the experimental arrangement of autolysis. He measured the undigested protein of the extracts by a nephelometric method, after precipitation with sulphosalicylic acid and HCl. He apparently disregarded the activation by H_2S or by glutathione; and as regards the influence of heavy metals on proteolysis, he found for some of them an inhibiting action, for others a promoting one, which is not in agreement with the ideas of Krebs. A rat tumour (Cori-tumour) showed nearly the same behaviour as the liver of the same species. A clear review of the subject is due to Kleinmann [1931], who emphatically denies any special feature of proteolytic enzymes in tumours.

However, the idea that proteolytic processes should be very pronounced in tumours cannot be lost sight of, as we find also in recent times observations pointing to strong digestive action of tumour cells (Rous sarcoma) *in vitro* on fibrin and also on normal tissues; and every histologist knows the large necrotic, *i.e.* autolytic, processes in tumours, chiefly in those of the small rodents. I may point to the recent work of Price [1931], who was not able to confirm the ideas of Robin [1919], that in malignancy normal surrounding tissue is digested and that the growth builds up its protein from the digestion products; but he found a larger content of soluble N (non-protein-N) in the growth (Brown-Pearce tumour of rabbit) than in the adjacent normal tissue, into which autolytic products diffuse *post mortem*. He concludes: "evidence has been obtained that a malignant growth does not liberate proteolytic enzymes into the surrounding tissues"; but he does not deny the extensive proteolysis in the tumour itself.

Experimental.

Proteinase. General features of action.

The present work is to be considered as an attempt to study the proteolytic function of a tumour (Ehrlich's strain of mouse cancer) in a more natural way than that used by previous investigators, *i.e.* in a way more related to the technique of Stern for normal cathepsins. Like this author, I let the tumour enzymes react on the proteins of the tumour itself (autolytic function): the extracts, prepared according to Waldschmidt-Leitz and Deutsch [1927] with acidified glycerol (one part by weight of fresh tissue, minced and mixed with two volumes of 87 % glycerol acidified with 0·15 % of *N* acetic acid, kept for some hours at 37°, filtered through paper, and kept in ice-box), were diluted with water, brought to a proper p_H and placed in an Ostwald thermostat at 37° for the required time. The enzymes, which are inactive at a high concentration of glycerol and at low temperature, are able to act on the

proteins (about 0·5 % in tumour extracts prepared as above), when the glycerol reaches a high dilution (5 %) and when the system is brought to the proper temperature and reaction. We may then make an estimation of the rate of digestion by many methods. Stern used the nephelometric estimation of the undigested proteins, which seems to me not very reliable, as the dispersion of the precipitate obtained with HCl and sulphosalicylic acid is very variable and therefore the degree of turbidity may not run parallel with the content of undigested protein. I suppose that some nephelometric observations express more the influence which some factors (p_H, foreign substances, *etc.*) exert on the dispersion of proteins precipitated by sulphosalicylic acid than the different degree of protein digestion; at least these two different ways of modifying the turbidity may show themselves in the final result. Besides this a nephelometric method must be excluded when we wish to try the influence of a turbid solution (such as lipoids generally give) on digestion. On the other hand all methods consisting in the estimation of digestion products, and particularly the Van Slyke gasometric method, require rather large quantities of substrate to give sensitive and reliable results; and the digestion must generally last many hours (24) in working with organ enzymes, so giving rise to some disturbing conditions.

After many attempts I found it to be a sufficiently exact method to weigh the protein precipitate obtained with trichloroacetic acid (final concentration, 5 %). This precipitate (undigested protein) is collected on previously weighed filters, thoroughly washed with dilute trichloroacetic acid, then with alcohol (hot and cold) and with ether, dried at 105–110° and weighed.

An example of one of the first experiments is given here.

Exp. 1. Three similar mixtures were made each containing

2 cc. of *M* buffer solution (sodium acetate-acetic acid) at p_H 3·8 + 36 cc. redistilled water + 2 cc. of a tumour glycerol extract (as above).

To mixture (1) 40 cc. of a 10 % trichloroacetic acid solution were at once added.

Mixture (2) remained 2 hours at 37° (water-bath); and then 40 cc. of 10 % trichloroacetic acid solution were added.

Mixture (3) remained 6 hours at 37° and then the same addition was made.

The three precipitates (undigested protein) were collected and washed, then dried and weighed. Results in mg. protein:

From mixture (1) 11·2 (starting-point : original protein content).
From mixture (2) 9·8 (extent of digestion = 12·4 %).
From mixture (3) 5·0 (extent of digestion = 55·5 %).

The final experimental method was generally arranged with 60 cc. total volume of the digestion mixtures, containing 3 cc. of the glycerol extract, 4 cc. of the buffer along with redistilled water and the substance, the action of which was to be tested on digestion. At the moment of stopping the digestion 20 cc. of a 20 % solution of trichloroacetic acid were added. The digestions

were always carried on at 37° (water-bath). When I wished to make a control by Van Slyke's gasometric method 10 cc. were removed from the mixture and treated in the usual apparatus, the remaining 50 cc. being used for the gravimetric method.

The different tumour extracts (old well-developed tumours or young tumours; more or less of necrotic areas; differently treated or untreated tumours) were not always quite alike as regards content of enzymes and of proteins; they showed also some differences in p_H-optimum, which in some cases changed with the age or time elapsed from the preparation. We know from the work of Willstätter and his school that the p_H-optimum of an enzyme depends on many factors.

A tumour extract, made in March 1932 from well-developed (aged 21 days) tumours of 20 mice, gave the following results after 10 days and after 60 days from the preparation (1 tissue + 2 acid glycerol).

Exp. 2. Digestion mixtures of 60 cc. total volume as described above containing 3 cc. of extract. Extent of digestion after 6 hours (%) at 37°.

	Extract 10 days old	Extract 60 days old
p_H 4	0	29
p_H 4·7	29	20·2
p_H 5·17	37	17·5

This extract acted well in the beginning at p_H 4·7–5·1, although the latter value was to be considered not very reliable because some precipitation spontaneously occurred in the mixture after 6 hours' incubation; I therefore took as optimum p_H 4·7, which was used in most of the experiments. Krebs worked at the same p_H in his important investigations. The more acid reaction (p_H 3·7–4) has often given very good digestion, as in Exp. 1; but sometimes in fresh extracts inexplicable inhibitions occurred (generally not so strong as in the case given above).

The comparison between fresh and older extracts was all the more difficult in that the amount of protein itself changed somewhat (slow spontaneous breakdown in spite of the high glycerol concentration). The extent of digestion had always to be referred to a new control value of undigested protein.

Table I.

Extent of digestion at 37° (%).

Extracts tested:	2 hrs.	4 hrs.	6 hrs.	12 hrs.
A at p_H 3·8	12·4	—	55·3	—
A at p_H 4·7	—	—	34·6	53·5
A at p_H 4·7 (1 month later)	—	—	29·5	—
A at p_H 4·7 (2 months later)	—	—	13·43	—
L.S.C. at p_H 4·7	—	—	29	—
L.M.C. at p_H 4·7	—	—	55·6	—
L.M.C. (19 days later)	—	—	11·7	—
R.	11·5	20·4	35·5	45
R. 2	9·5	18	32	40·2
R. 3	13	25	52	59·5

As regards the course of digestion, I may state that there are differences between the various extracts: if we take the average course at p_H 4·7 (the one usually adopted), we find 10–15 % digestion in the first 2 hours, 30–35 % digestion after 6 hours, and a final value of 50–60 %, even after 24 hours at 37°.

The behaviour of some different extracts is summarised in Table I.

These results are similar to those obtained by Stern [1931, Fig. 5] on a Cori-tumour of the rat.

Activation with H_2S and cysteine.

After passing a current of H_2S through the digestion mixture at room temperature for half an hour, no clear activating influence of H_2S could be detected by precipitation of the undigested protein. The estimation of precipitated protein by weight is rendered difficult in the mixture treated with H_2S by the precipitation of some sulphur, which may be largely removed by washing on the filter with carbon disulphide.

The result with 0·005 *M* cysteine hydrochloride was more definite. An extract gave an 82 % digestion in the presence of cysteine after 2 hours at 39° against 51·7 % in the control mixture without the amino-acid. Another experiment gave the following result.

Exp. 3. Three mixtures were prepared each containing 4 cc. buffer solution at p_H 4·7 and 3 cc. tumour extract (aged 23 days), made 1 : 4 with acid glycerol; to No. 3 were added 3 cc. of 0·1 *M* cysteine hydrochloride (Roche).

Mixture (1) was at once treated with 20 cc. of 20 % trichloroacetic acid; mixtures (2) and (3) remained 7 hours at 37° and were then treated with trichloroacetic acid.

Results: mg. of undigested protein:

From (1) 5·2.
From (2) 4·6 (11·5 % digestion).
From (3) 2·6 (50 % digestion).

Other experiments with extracts more active in themselves gave less striking, but still clear results. Cysteine is able to increase the activity of cathepsin in tumours as well as in normal tissues; and this influence may be observed also using as substrate the protein of the extracts themselves, contrary to the statement of Kleinmann.

Effects of lecithin.

Here also difficulties occurred in the estimation of undigested protein, because the phosphatide remained strongly adherent to the precipitate and a very long, tiresome washing with alcohol and ether was necessary.

Exp. 4. Six mixtures were prepared (total volume 60 cc.) each containing 4 cc. buffer solution at p_H 4·7; 3 cc. tumour extract A (made 1 : 3 with acid glycerol). To Nos. 1, 2 and 3, 3 cc. of a 1 : 3 dilution of ethyl alcohol in redistilled water were added and to Nos. 4, 5 and 6, 3 cc. of a 1 : 3 dilution in

redistilled water of a 2 % alcoholic solution of lecithin (Merck); *i.e.* 0·02 g. of lecithin were present in the mixture.

Mixtures (1) and (4) were at once treated with 20 cc. of a 20 % solution of trichloroacetic acid.

Mixtures (2) and (5) remained 6 hours and mixtures (3) and (6) 19 hours at 37°, and were then treated with trichloroacetic acid.

Results: mg. of undigested protein:

From (1) 15.
From (2) 9·8 (34·6 % digestion).
From (3) 7 (53·5 % digestion).
From (4) 14·2.
From (5) 7·8 (45 % digestion).
From (6) 6·4 (54·9 % digestion).

From this experiment a slight accelerating effect of the lecithin may be deduced. In other cases the effect was still less evident but never quite negative or inhibiting. It may be remembered that Pianese [1917] assumed an activating influence of lecithin on intracellular enzymes in cancer to explain his histological observations.

Dipeptidases and aminopolypeptidases.

The action of tumour extracts on dipeptides and polypeptides was investigated according to the usual methods of Waldschmidt-Leitz and co-workers.

The dipeptidases were tested on leucylglycine (from the laboratory of Prof. Waldschmidt-Leitz, Prague); and the aminopolypeptidases on leucylglycylglycine (Roche). The solutions of the peptides were prepared as follows.

3·764 g. of leucylglycine or 4·9 g. of leucylglycylglycine were dissolved in 40 cc. of a 1 : 1 mixture of *N* solutions of ammonium chloride and ammonia; 20 cc. of distilled water were added, the dissolution being helped by warming. 5 cc. of *N* acetic acid solution were added and the volume made up to 100 cc. with water. These solutions (p_H 8) contain 0·001 *M* peptide.

For the experiment 5 cc. of peptide solution were mixed with 4 cc. of redistilled water and 2 cc. of extract; 5 cc. were at once taken and used for a blank estimation, and a second estimation was made on 5 cc. after 1 hour at 37°. The estimations were made according to the alkalimetric method of Willstätter and Waldschmidt-Leitz [1921] in alcohol (5 cc. of digestion mixture were added to 100 cc. of neutralised absolute alcohol and titrated with alcoholic 0·1 *N* KOH with phenolphthalein as indicator). The extent of digestion is given by the increase of carboxyl groups set free and therefore by the difference between the estimations before and after digestion (cc. of 0·1 *N* KOH).

Three extracts from cancer of mice were investigated as to content of dipeptidases. In each group of mice the livers were also taken and extracts with acid glycerol prepared and tested in the same way, so that a comparison of peptidase content in tumour and in liver is possible.

Results. Dipeptidase contents (cc. of alcoholic 0·1 *N* KOH).

Extracts (set of mice)	Tumour	Liver
L.S.C.	1·60	2·90
L.S.	2·15	2·65
L.M.	0·70	0·65
Average	1·48	2·06

As regards aminopolypeptidase I tested only an extract from the tumours of 5 mice submitted to a treatment with lecithin (Merck) and found none at all. The livers of the same animals showed a content corresponding to 0·6 cc. of 0·1 *N* KOH.

My results may be well compared, on the ground of the identity of methods of estimation, with the results of Waldschmidt-Leitz and Schäffner [1930] on a rat sarcoma: they found values of dipeptidases which varied from 1·12 to 1·50 cc. 0·1 *N* KOH and averaged about 1·30. My results were very similar, but slightly higher; the epithelial nature of the tumour investigated by me may account for the small difference. I cannot state whether there is an increase with progression of tumour growth, because I examined in every case well-developed cancers. There may be some importance in the complete absence of aminopolypeptidases in contrast to the high content found by the investigators just mentioned, and also to the content of the liver of the same animals. This point deserves further research.

Attempts to modify the enzymes of tumours by treatment of the tumour-bearing organism.

Some of the foregoing data were drawn from two experiments bearing on the effects of lecithin treatment of tumours. In a long series of papers from 1926 onwards I had tried to study the influence of many lipoid fractions on the growth of tumours, and many experiments pointed to an inhibiting action of lecithin, parenterally introduced during the development of mouse cancer [1930; 1931]. As I had the opportunity a few months ago of using a distearyl-lecithin prepared by Grün and Limpächer [1923] in my investigations [1932] on the effects of phosphatides on proteolytic enzymes, and was able to obtain more of this interesting compound through the kindness of Dr Limpächer, I submitted some cancerous mice to treatment with it. The small quantity of synthetic lecithin at my disposal was insufficient for the treatment of a large series of mice.

Of 11 cancer-bearing mice (graft on May 10th) 5 were kept as control; 6 received every two days from May 17th till 27th an injection of 1 cc. of a 1 % solution in water of the synthetic lecithin, which after thoroughly grinding and by gentle warming gives a stable solution in distilled water. Every mouse received 0·06 g. in the course of the 10 days' treatment. By weighing the tumours of the untreated and treated mice (which were all killed on the 27th) and dividing the weight of every tumour by the number of days elapsed since the grafting, we get the growth index or daily increase of tumour mass.

The average growth index was 0·084 g. for the treated mice; 0·138 g. for the untreated. The synthetic phosphatide gave therefore an inhibition of the tumour growth of about 39 %.

Extracts were prepared from the tumours of both groups of mice, taking great care to work quantitatively and to obtain two equally diluted preparations.

They were tested as to catheptic (proteinase) function in the usual way and as to dipeptidase content; the livers were also worked up and tested. Some of the data given before were drawn from this experiment; they may be summarised here as follows.

Tumour extracts from untreated mice gave a protein autolysis of 29 % in 7 hours, and a dipeptidase content of 1·60 cc. 0·1 *N* KOH.

Tumour extracts from treated animals showed less proteolytic activity (20 %) and more dipeptidase content (2·15 cc.).

Liver extracts gave quite opposite results: the catheptic function on their own proteins seemed to be a little higher (with some uncertainty) in treated mice; the dipeptidase function was here a little reduced. At any rate the differences were smaller than in the tumours.

Another experiment was undertaken with lecithin *ex ovo* (Merck), which had to be dissolved in alcohol (2 %), the solvent evaporated on the water-bath and the fatty material taken up with water for injection. The inhibiting action on cancer of a treatment with a total of 0·05 g. for each mouse was less pronounced here than in my previous researches [1930], when I injected lecithin together with pig-serum: 23·7 % inhibition was observed in 6 treated mice against 4 untreated. The estimation of the proteolytic function gave a lessened activity in tumours from treated mice, just as in the experiment with synthetic lecithin (30 % digestion in 7 hours against 50 %). Dipeptidases were not compared for lack of material.

It may be said that in two experimental series dealing on the whole with 20 mice and with 4 extracts from their tumours, a treatment with lipoids, which was able to restrict the growth of a very virulent tumour, also reduced the content of proteinases or rather modified the conditions of the system proteinase *plus* protein of tumour tissue in a way which led to a lowered digestive activity. The enzymes of a normal organ (liver) seemed to be less and quite independently affected.

Discussion.

These experiments are favourable to the opinion that the proteinase and "ereptase" activities in tumours do not differ essentially from those already well known in normal tissues. At least I was not able to discover a single specific feature. Quantitatively considered, they are to be ranged rather high, although not higher, and sometimes indeed lower than the corresponding functions of some normal organs. I would point out that it is not right to compare an enzymic function of a tumour with the same function in any normal tissue other than that which itself gave origin to the tumour.

That proteolysis has an importance in tumours during life is well shown by the high digestive power of tumour cells *in vitro* (Rous sarcoma) and by the very common observation of large autolytic and necrotic areas. However, the relation to cell multiplication and to growth is not clear. I may state that in two sets of experiments a decrease of growth velocity seemed to be accompanied by a decrease of proteinase content and once by an increase of dipeptidase; but it would be imprudent to lay too much stress on such differences between extracts from parallel groups of anmals. I may rather state that the results of *in vitro* experiments are often the reverse of those of *in vivo* experiments. Thus while lecithin increases the proteolytic activity of extracts from normal organs on gelatin, as I found before [1932], and also of tumour extracts on their own proteins, as is shown in this paper, lecithin itself, the synthetic non-physiological compound as well as the preparation *ex ovo*, diminishes the growth of tumours and the autoproteolytic activity of the tumour tissue. The lecithin therefore influences the tumour not only or not chiefly directly, but also indirectly, through the metabolism of the tumour-bearing organism. The content of proteinases and dipeptidases in the tumour may change under a lecithin treatment of the animals independently of the content in a normal organ (liver). The proteinases as studied in an autolytic experiment need no special activator: they are active by themselves in tumours as well as in normal tissues and produce in 6 hours an average digestion of 35 % of the tissue proteins. In other words the extracts prepared with acid glycerol [Waldschmidt-Leitz and Deutsch, 1927] contain a complete enzymic system. However, some substances like phosphatides and cysteine can enhance the digestive activity more or less. These substances are not necessary activators, but rather accelerators of the enzymic function. It may be that compounds containing the —SH group are already present in the extracts and work as true activators; then the added cysteine would complete the supply of it. And it may also be admitted that the different degrees of activity of the extracts depend on the amount of natural activators, *e.g.* glutathione, already present. But I have derived no direct proof from my experiments of such a general constitution of the enzymic system in tumours. Some experiments would suggest that cysteine, which generally facilitates the proteolysis, is able under not well defined conditions to reverse this function and to produce a new reconstitution of protein. As the experimental demonstration of this very important phenomenon is not yet conclusive, I preferred not to report the experiments and to reserve this point for further investigation.

I would conclude that the strong proteolytic functions, often exhibited by living tumour cells, are not the expression of special characteristics of the enzymic equipment of the cell but rather a consequence of the biological derangement, which allows the cell to use its normal enzymes in an abnormal way. As with many other aspects of malignancy we must look at the vital behaviour more than at the purely chemical constitution of cells. As the circulation in tumours is somewhat defective, it may happen that some

derivatives formed by the breaking down of the very labile tumour cells are not removed so readily as in normal tissue; and they may on the one hand check the further disintegration (which we know is possible chiefly from the work of Rona *et al.* [1930], but also on the other hand sometimes facilitate proteolysis, if, among the products of the disintegration of cellular lipoids, proteins and lipoid-protein compounds, substances predominate which may enhance the catheptic function, as do lecithins and cysteine.

A very complicated situation arises which cannot be expressed by a general law and explains the differences shown by tumours of different origin and age or submitted to some treatment.

Summary.

1. The cancer of mice (Ehrlich's strain) gives extracts with acid glycerol, which may be easily investigated as to proteolytic activity on their own proteins (autolytic experimental type). The general features of the tumour proteinases have been described. The optimum p_H changes a little according to the age of tumours and of extracts; generally the digestion experiments were carried on at p_H 4·7. The activity of proteinases in tumours is not higher than in some normal organs, although an exact statement on this matter (comparison with the organ from which the tumour growth started) is not easy. The digestion averages generally 35 % of the extract protein in 6 hours at 37°.

2. Lecithin has a slight accelerating action on proteolysis.

3. Cysteine has a more or less pronounced accelerating action.

4. A variable content of dipeptidases was found; aminopolypeptidases were lacking in the one series examined, while the livers of the same mice contained them.

5. Treatment of the tumour-bearing mice with synthetic lecithin and with lecithin *ex ovo* delayed the tumour growth, and produced some modifications in the enzyme content, which are briefly discussed. These modifications do not run parallel with modifications of enzymes in normal organs (liver).

6. The proteolytic enzymes (cathepsins) in the investigated tumour show no special features in comparison with the enzymes of normal tissues, although their function in the living tumour cells may be in some way peculiar. The malignant cell may utilise a normal enzymic equipment in an abnormal way.

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CLXXVI. THE PROTEIN COMPONENT OF THE ERYTHROCYTE MEMBRANE OR STROMA.

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Our chemical knowledge of the structural moiety of the red blood corpuscles is very scanty, particularly when compared with the thoroughness with which the haemoglobin has been studied. This lack of knowledge contrasts strongly with the keen interest, which has long been directed to the study of haemolysis, agglutination and permeability phenomena, for which the erythrocyte membrane is of the utmost importance.

Concerning the structure of the mammalian erythrocyte the view already advanced by Leeuwenhoek in 1719 *viz.* "*vesiculam aqua plenam habemus*," seems to be that of the present day also. According to this view the inner haemoglobin sol is surrounded by a kind of membrane, which after the loss of its fluid content forms the ghosts or "Blutschatten." This question is thoroughly dealt with in Weidenreich's monograph [1903]. Since that time the term stroma has generally been used in the sense of membrane, although the name was originally introduced by Rollett in 1871, to designate the trabecular framework in the meshes of which Brücke in 1861 and Rollett supposed that the haemoglobin solution was held. It is beyond the scope of this investigation to discuss more closely the histological details in the structure of the mammalian erythrocyte. The main purpose is to study the protein moiety of the membrane or stroma.

The lipoids of the red blood corpuscles have been thoroughly studied both from the qualitative and the quantitative points of view. It is, however, impossible to find any detailed information in the literature about the protein component. Thus Wooldridge [1881] and Halliburton and Friend [1889] describe only some properties of the stromata, the latter state that they contain a globin similar to that of haemoglobin and that no phospho- or nucleo-proteins are present. No information is gained from the work of Beumer and Bürger [1912], and their preparations contained 50–70 % haemoglobin. A more recent paper by Haurowitz and Slàdek [1928] deals with analysis of stroma preparations, containing from 0·07 to 0·16 % iron. This admixture of haemoglobin caused their figures for the content of arginine-nitrogen to vary between 4·2 and 10·4 % of the total nitrogen and those for the histidine-nitrogen from 3·2 to 10·4 %.

For several reasons, however, it is essential to know the nature of the stroma protein. First, it is known that the acid proteins are strong antigens, as is the erythrocyte membrane, whereas haemoglobin and the basic proteins have very weak antigenic properties, if any at all. Furthermore, a most elaborate theory of the permeability of ions through the erythrocyte membrane is founded on the assumption that the membrane contains a basic protein, some kind of globin similar to that of haemoglobin.

The fact that the erythrocyte membrane is non-permeable to cations has been explained by assuming that it contains a basic protein, which with its positive electric charge would prevent the passage of the cations. As has been shown by Michaelis and his co-workers in a series of publications [1925; 1926; Fujita, 1926] the permeability of artificial membranes to ions is largely influenced by the electric charge of the membrane, if the diameter of the pores is small enough. When a membrane with pores of a suitable size separates two solutions containing the same salt in different concentrations, the diffusion of the cations through the membrane is prevented, if the membrane has a positive charge, and that of the anions, if it has a negative charge. Thus the non-permeability of the erythrocyte membrane towards cations could be easily explained, if the membrane contained a layer of a basic protein. Mond [1927] brought forward some evidence in favour of this view. In a slightly alkaline solution of p_H 8·3 the system according to him is reversed and the membrane is permeable to cations but not to anions. The change occurs at the supposed isoelectric point of globin, where the charge is reversed. Hence Mond [1928] makes the following statement: "Danach besteht die Grenzschicht der Erythrocyten aus basischen Eiweissphasen (Globin) und Lipoidphasen, die mosaikartig in ein Stromagerüst eingelagert sind." He admits however that an acid protein may also occur in the membrane contributing to its electric charge, which is known to be negative [Höber, 1904].

The separation of the membranes of the red blood corpuscles.

The membranes may be flocculated out after haemolysis either with immune serum or with acid. Precipitation with acid is the oldest and most generally employed method. As early as 1863, in W. Kuhne's text-book of physiological chemistry, a method is given of separating the membrane by passing a stream of carbon dioxide through the blood after haemolysis. Hoppe-Seyler and Wooldridge precipitated the membranes by means of acid potassium sulphate, which they believed to shrink the membranes, but they did not refer to the acid character of the salt. Later the method of introducing carbon dioxide after haemolysis with distilled water and the removal of the leucocytes was used by Abderhalden, Bang, Beumer and Bürger and others. In the present investigation the membranes were sometimes flocculated with immune serum, but this was mainly done by the addition of acid.

Attention was at first given to the possibility that the substance precipitated with the acid might be an artificial product. The haemoglobin might, as soon as it became positively charged on the acid side of p_H 6·8, have been precipitated together with negatively charged lecithin, just as the nucleoproteins are artificial products, *i.e.* salts of negatively charged nucleic acids with positively charged proteins [Hammarsten, 1924]. In that case, the protein component of the membrane would have the qualities and composition of haemoglobin. However, analyses of preparations with even a high haemoglobin content showed that such was not the case.

In view of the desirability of precipitating the membranes at the reaction of the blood, antisera were prepared by the injection of blood corpuscles of horse into goat and rabbit. When immune serum was added to the extent of 1–5 % to the haemolysed horse blood, even without the addition of sodium chloride, a precipitate was obtained which could be removed by centrifuging. Alternate washing in distilled water and physiological salt solution produced a considerable fading, but thorough separation of the haemoglobin was achieved only occasionally.

The old method of using carbon dioxide after dilution with several volumes of distilled water was also not found to be advantageous. The precipitate was not easy to filter through a cloth, owing to the fact that carbon dioxide does not give a sufficiently high degree of acidity. In four different preparations after passing carbon dioxide through the solutions for half an hour, p_H values of 6·03, 6·15, 6·10 and 6·09 (quinhydrone) were obtained. Even if a flocculation took place in haemolysed horse blood at p_H 6·20 (possibly 6·40) the precipitate was easiest to separate from the mother-liquor at p_H 5·6–5·1. Furthermore, as the carbon dioxide escaped, the precipitate became more difficult to handle. Since bisulphite solution easily gave rise to the development of methaemoglobin, precipitation by mineral acid was selected as the most suitable method. If 0·1 to 0·01 *N* HCl is added with rapid stirring, there is no danger, owing to the strong buffering effect of the proteins, that the reaction will be too acid. The flocculation of the membranes in horse blood is optimum at a p_H of about 5·5. If the preparation is carried out at a low temperature, there is very little risk that the haemoglobin will be denatured. According to Booth [1930] haemoglobin is denatured at 0° instantaneously at p_H 3, and in 10 minutes to 50 % at p_H 3·5. At p_H 5·5, the risk of denaturation therefore should be insignificant.

As far as haematin development is concerned, Svedberg and Nichols [1927] maintain that this does not take place in carbon monoxide haemoglobin when the p_H is between 6·0 and 9·05. When acid is added to haemolysed horse blood, no change in colour is seen until the p_H is 4·3. When it is 4·0, the change is more distinct. No haematin formation need therefore be apprehended when the range of p_H is between 6·0 and 5·5.

On haemolysis by dilution with distilled water, there is the risk that any globulin present in the corpuscles might be precipitated and mixed with the

corpuscle membrane. Dilution with distilled water and adjustment of the p_H to between 5 and 6 give all the pre-requisites for precipitation of the globulins. It was therefore necessary to prove that the precipitate obtained on the addition of acid to blood haemolysed with distilled water belongs to the membrane.

A strong argument in favour of this supposition is the very fact that no further precipitation is obtained with acid after removal of the precipitate obtained with immune serum. Presumably the immune serum acts on and flocculates the membranes.

Another argument is the fact that the membrane can be removed by passing the liquid through a Seitz filter. Blood haemolysed with distilled water and not entirely transparent becomes quite clear when passed through a Seitz filter and gives no precipitate on the addition of acid to the filtrate. Possibly existing globulins would in no case have been quantitatively precipitated at the reaction of the blood and after passage through the filter a turbidity would appear when the p_H was adjusted to 5·4 or 5·6. The conclusive proof, however, is given by certain experiments with saponin haemolysis. If the paste of corpuscles is diluted with saline instead of with distilled water and haemolysis is produced with saponin, no globulins should be precipitated. Here, too, the liquid is not quite clear, but becomes so after passage through the Seitz filter. Before filtration, the addition of acid to the blood, haemolysed with saponin, results in the characteristic precipitation, but after it there is no clouding or opalescence. It is thus clear that the precipitate herein regarded as the protein component of the erythrocyte membrane, obtained by the addition of acid to the haemolysed blood, does actually originate from the membrane and not from any protein present in solution within the blood corpuscles.

When precipitation had been obtained with acid, it was necessary to wash the membranes free from haemoglobin. With this object in view, the precipitate was chilled and filtered through silk. When it was again suspended in cold water the precipitate could usually be more easily filtered. Continued washing with distilled water in a centrifuge caused only a slight removal of haemoglobin from the precipitate. Though the last washing might be quite colourless the precipitate was usually still red and preparations made from it contained between 30 and 60 % haemoglobin. The treatment employed by some authors with a dilute carbonate solution, followed by reprecipitation with acid, had a certain purifying effect in some cases, but also easily caused methaemoglobin formation.

Faced with the need of finding some other means, I tried, after washing with distilled water, to dissolve the haemoglobin in an acid buffer solution. The assumption was made that the protein of the membrane itself must have its isoelectric range on the acid side of the neutral point, more exactly at a p_H between 5 and 6 for horse blood. Its passage into a more coarsely dispersed phase and its flocculation on the addition of acid must evidently be

due to a diminished electrolytic dissociation. This is also corroborated by the fact that the precipitate disappears again on the acid side of the given p_H range. If this were the case, the risk of loss through washing in a buffer solution of a suitable acidity would be slight, while the haemoglobin at a p_H of, for instance, 5·5 would be more extensively dissociated than in distilled water. A phosphate buffer with the acidity given above was chosen and the effect was striking. After the membrane precipitate had been suspended in this buffer solution, vigorously shaken with pieces of ice and glass beads and then centrifuged, the mother-liquor turned very red and the membrane precipitate was pale. This washing was repeated a few times. It was important to change the concentration of the phosphate in the washing fluid occasionally, though without changing the p_H. In this way it was possible to get almost colourless preparations. In the first preparation made in this way, the haemoglobin content, ascertained by iron analysis, fell through a single washing with the buffer solution from 55 to about 15 % of the dry substance. The precipitate used in this case had not given up any further haemoglobin to distilled water. This shows plainly that the iron in such carefully treated preparations is contained in the haemoglobin and is not present to any significant degree in any other form, as Beumer and Bürger implied.

The method of isolation employed.

Two litres of fresh, defibrinated horse blood are filtered through gauze and the corpuscles washed with cold physiological salt solution at least 4 times in a centrifuge taking 2 litres. After washing, the tubes are left standing overnight in the refrigerator. Next morning, 6 volumes of cold distilled water are added, after which the leucocytes are centrifuged away at a speed of 2400 revolutions per min. for 20 minutes. This amount of water is, according to Ponder [1925], required for complete haemolysis. According to Abderhalden and Deetjen [1907], the leucocytes are removed from the plasma when centrifuged for 15 minutes at 1500 revolutions per min. They remain in a sticky layer at the bottom of the centrifuge-tubes, from which the liquid can be decanted off. The smallest amount of acid is then found, which gives the optimum of flocculation. For the whole quantity, 80 to 100 cc. *N* HCl are usually required. When the liquid has been chilled with ice, it is poured into 25 litres of cold distilled water. The acid is diluted with 2 litres of distilled water in which ice is placed and slowly added by vigorous stirring to the 25 litres. The whole is then poured into large glass cylinders in the cold room, where the membranes settle. The clear liquid is removed and the precipitate collected on pieces of silk. The latter process takes about 2 hours. The voluminous precipitate is suspended in 10 litres of distilled water and the liquid this time runs easily through the silk. The precipitate should be allowed to drain thoroughly. It is suspended in 1·5 litres of distilled water together with pieces of ice and glass beads and is pressed through a tightly stretched filter-cloth (No. 40) by scrubbing with a hard brush. This is a very important process.

Without such mechanical treatment the washing will not be effective. After this the precipitate is centrifuged for 15 minutes. The clear liquid can be poured off. The precipitate is suspended in 1·5 litres of buffer solution (prepared from 15 cc. stock solution to 1·5 litres distilled water) and shaken vigorously with pieces of ice and glass beads. After centrifuging the washing is repeated in the same way as long as haemoglobin is removed. It is important that the later washings should be made with buffer solutions alternating with 15 cc. and 30 cc. stock solution to every 1·5 litres cold water. If the preparation is successful, the membrane protein is almost colourless. If too long a time has been spent on the preparation the brown colour of methaemoglobin often appears. The preparation after haemolysis should be completed in the course of 4 or 5 hours. In order to remove some impurities introduced with the ice, the precipitate is suspended at a low temperature in cold distilled water and 0·1 *N* sodium hydroxide added until the reaction is slightly alkaline to litmus but still acid to phenolphthalein. The impurities are centrifuged off together with some denatured protein. An addition of the same amount of 0·1 *N* hydrochloric acid re-precipitates the membrane protein. It is allowed to settle, after which it is centrifuged off, and dried with cold alcohol and ether.

The yield in the different preparations varied considerably, *viz.* between 1·0 and 2·5 g.

The stock buffer solution is prepared by the addition of a little secondary sodium phosphate to an almost saturated solution of primary potassium phosphate, after which the p_H is adjusted colorimetrically by the addition of suitable amounts of acid or alkali.

Analysis of the membrane preparations.

As stated above the preparations were washed with alcohol and ether. In order to avoid haematin formation no washing with hot alcohol was made. In spite of this the lipoids were practically completely removed as found on analysis of one of the largest preparations.

The iron and nitrogen contents of some of the stroma preparations are given in Table I, calculated on dry substance (115° *in vacuo*).

Table I.

Preparation ...	1 Horse	2 Horse	3 Horse	4 Sheep	5 Sheep	6 Horse	7 Horse	8 Horse
Ash (%)	0·45	0·89	—	—	—	—	—	—
Nitrogen (%)	14·83	14·16	14·01	14·75	14·71	14·64	15·40	15·29
Fe (%)	0·047	0·049	0·048	0·058	0·064	0·069	0·091	0·136

Purified samples of some other proteins of the blood were also analysed. The oxyhaemoglobin was recrystallised twice. Its nitrogen content calculated on dry substance was 16·80 %. The figure given by Mitchell and Hamilton [1929] is 16·86. Fibrin from ox blood was washed free of haemoglobin, defatted and dried with alcohol and ether. Its nitrogen content was 16·82 %

calculated on ash-free dry substance (16·91 Mitchell and Hamilton). Serum-globulin from horse blood was reprecipitated with ammonium sulphate twice and dialysed, first against tap water and then in the electrodialyser of Pauli. Its nitrogen content was 15·46 % calculated on dry ash-free substance.

The nitrogen distribution according to Van Slyke.

The procedure of Van Slyke was performed in the usual way. For the sake of comparison the other blood-proteins except serum-albumin were analysed with the same technique.

5 g. air-dry substance were hydrolysed by boiling in 200 cc. 20 % HCl for 48 hours. After removal of the excess of HCl the hydrolysate was diluted to 200 cc. and its nitrogen content determined. The solution was divided into two parts, one of 100 cc. and one of 92 cc. (88 cc. in one case), which were analysed separately for amide-N and humin-N and precipitated with phospho-tungstic acid. The arginine analysis was made according to the modification of Plimmer and Rosedale [1925]. A special apparatus was used. The boiling flask was fitted to the cooler directly by a ground connection and washed air was taken in by suction according to the modification of Koehler [1920]

Table II. *Nitrogen distribution according to Van Slyke in some blood-proteins.*

	The stroma protein	Fibrin	Serum-globulin		Oxyhaemoglobin	
g. air-dry substance	2·5	2·5	5		5	
Volume after hydrolysis cc.	100	100	200		200	
Nitrogen in 4 cc., mg. (Kjeldahl)	12·85					
	12·80	14·27	13·78		15·16	
	13·00	14·31	13·81		15·16	
Av.	12·88	14·29	13·80		15·16	
Total nitrogen, mg.	322	357·3	690		758	
Volume taken to analysis, cc.	88	92	100	92	100	92
Amide-N, mg.	26·37	32·77	33·22	30·92	22·14	20·59
Amide-N % of total N	**9·30**	**9·97**	**9·63**	**9·75**	**5·84**	**5·91**
Humin-N, mg.	8·89	9·97	6·27	5·90	11·74	10·89
Humin-N % of total N	**3·15**	**2·17**	**1·82**	**1·86**	**3·10**	**3·12**
Monoamino-N in 25 cc., mg.	16·95					
	16·90	19·24	21·42	20·08	21·85	19·71
	17·00	19·29	21·57	20·18	21·90	19·58
Av.	16·95	19·27	21·50	20·13	21·88	19·65
Monoamino-N % of total N	**59·81**	**58·62**	**62·32**	**63·42**	**57·73**	**56·36**
Diamino-N in 10 cc., mg.	7·52	9·42	8·73	7·99	11·88	11·03
	7·62	9·43	8·88	7·91	11·90	10·96
Av.	7·57	9·43	8·81	7·95	11·89	11·00
Diamino-N % of total N	**26·71**	**28·69**	**25·54**	**25·05**	**31·37**	**31·55**
Nitrogen recovered, %	98·97	99·45	99·31	100·0	98·04	96·94
Arginine analysis on 30 cc. mg. N	6·19	7·70	6·35	6·19	4·64	4·41
	5·91	7·70	6·37	6·19	—	4·34
Av.	6·05	7·70	6·36	6·19	—	4·38
Arginine-N % of total N	14·23	15·58	12·29	13·00	8·16	8·37
Arginine in 100 cc., mg.	125·4	159·2	131·8	128·2	96·1	90·8
Arginine calculated on dry substance, %	**6·13**	**7·94**	**5·87**	**6·22**	**4·21**	**4·32**

through a glass tube fused into the upper part of the flask. The duplicates agreed very well as is seen from Table II. The figures found for haemoglobin are probably too high. The determinations of the amino-nitrogen were made in an all-glass micro-apparatus as recommended by Folley [1930]. This apparatus is a real improvement compared with the ordinary Van Slyke apparatus with its rubber connections.

The details and the results are given in Table II. The stroma protein analysed here is preparation No. 2 in Table I.

It is evident that the stroma protein shows no similarity to haemoglobin in its quantitative composition. The nitrogen distribution according to Van Slyke as well as the low nitrogen content indicates that it is an acid protein. There remained however an uncertainty in the analysis given in Table II, *viz.* the content of the mono-basic amino-acids. On calculating the histidine and lysine contents in the usual way from the figures found for the total N, amino-N and the arginine-(+ cystine-)N no concordant results were obtained. Hence these were left out and instead the arginine and histidine contents of the solution containing the basic amino-acids in 100 cc. were determined by means of the colour reactions of Sakaguchi and Pauly. 1 cc. of the solution was diluted to 75 or 150 cc. and analyses were performed as described in the next two papers. The results were as follows (see Table III):

Table III.

	Arginine found by the ordinary alkaline hydrolysis mg.	Arginine found with the Sakaguchi reaction mg.	% calculated on dry substance	Histidine found with the Pauly reaction mg.	% calculated on dry substance
Stroma protein	125·4	118·6	5·80	53·9	2·63
Fibrin	159·2	159·6	7·97	44·6	2·23
Globulin	131·8	130·0	5·82	47·9	2·14
	128·2	110·4	5·36	41·3	2·00
Oxyhaemoglobin	96·1	76·5	3·35	181·0	7·93
	90·7	69·0	3·29	160·0	7·62
				178·0	7·81
				160·0	7·62

The figures given for oxyhaemoglobin by Vickery and Leavenworth [1928], as found by the isolation method, were 3·32 % arginine and 7·64 % histidine, which are exactly the same as found here by the two colorimetric methods mentioned.

Even if the figures given here for the arginine and histidine content of the stroma protein are not to be considered as final and are to be confirmed by further analysis it is evident already that there is a striking difference between the stroma protein and haemoglobin.

The nitrogen distribution and the contents of arginine and histidine thus separate the stroma protein from the haemoglobin, but do not give any certain information about its relation to fibrin and globulin. On analysing the

different blood-proteins for tyrosine and tryptophan, the stroma protein is found to be different from all the other blood-proteins. Its content of these two amino-acids differs strikingly from that of fibrin and globulin as is seen in Table V. The main protein of the erythrocyte membrane or stroma is thus, if homogeneous, a protein *sui generis*, which for the sake of convenience could be called stromatin.

Table IV. *Tyrosine and tryptophan contents of the stroma protein as found by the Folin-Marenzi [1929] method.*

Calculated as % of dry substance.

Preparation No. in Table I ...		5 Sheep	4 Sheep	6 Horse	2 Horse	7 Horse	3 Horse
Iron content, % of dry substance		0·064	0·106	0·0687	0·049	0·091	0·048
Haemoglobin content: Fe % × 100/0·345		18·6	30·7	20	14·2	26·5	14
Tyrosine		3·17					
		3·08		3·10		2·91	
		3·08		3·11	2·90	3·02	
		3·08	2·78	3·15	3·02	3·00	2·90
		2·93	2·70	3·25	3·19	2·83	2·94
	Av.	3·09	2·74	3·15	3·04	2·94	2·92
Tryptophan		1·29					
		1·53					
		1·48					
		1·48			1·43		
		1·45	1·46	1·47	1·52	1·45	
		1·45	1·49	1·47	1·48	1·41	1·50
		1·39	1·51	1·46	1·44	1·47	1·56
	Av.	1·44	1·49	1·47	1·47	1·44	1·53

Table V. *Tyrosine and tryptophan contents of the blood-proteins as found by the Folin-Marenzi method.*

Calculated as % of dry substance.

		Fibrin (ox)	Serum-globulin (horse)	Serum-albumin (horse)	Oxyhaemo-globin (horse)	The stroma protein (horse and sheep)
Tyrosine		5·60	5·26			
		5·50	5·36			
		5·45	5·22			
		5·69	5·32	4·10		
		5·39	5·22	4·21	2·65	
		5·45	5·04	4·33	2·56	
		5·57	5·09	4·39	3·02	
		5·69	4·89	4·38	3·12	3·01
	Av.	5·54	5·18	4·28	2·84	
Tryptophan		4·28	2·12			
		4·18	2·26			
		4·21	2·18			
		4·32	2·24	1·03	1·28	
		4·04	2·29	0·99	1·25	
		4·11	2·15	0·95	1·29	
		4·01	1·95	1·08	1·24	
		4·35	2·46	1·05	1·25	1·46
	Av.	4·19	2·21	1·02	1·26	

THE AMOUNT OF THE STROMA PROTEIN PRESENT IN THE ERYTHROCYTES.

An attempt was made to determine approximately the amount of stroma protein present in the red blood corpuscles. Owing to the difficulty of removing the haemoglobin only indirect methods were tried. The membranes were either filtered off by passing the haemolysed blood through a Seitz asbestos filter or they were flocculated by means of immune serum or with acid. In each case nitrogen analyses were made of the haemolysed blood and of the fluid after removal of the membranes, due attention being paid to the dilution and to the nitrogen content of the immune serum. The loss of nitrogen per volume haemolysed blood was calculated as % of the total N content. The accuracy of the results depends largely on the nitrogen analyses. The error of the Kjeldahl method is not to be neglected, but it was found that other factors, which could not be controlled, exerted a much greater influence. Some typical experiments are given here.

Exp. 1. Corpuscles from horse blood, washed 9 times with saline, were haemolysed with 5 volumes of distilled water. The leucocytes were removed by centrifuging.

mg. nitrogen per 3 cc. of the haemolysed blood (Kjeldahl)

25·22, 25·17 and 25·19. Mean 25·19.

The liquid was pressed through the Seitz filter thus avoiding concentration through evaporation.

mg. nitrogen per 3 cc. after passage through the filter

24·40, 24·65, 24·50, 24·42. Mean 24·49.

Loss 0·70 mg. N per 3 cc. or 2·78 % of the total N before filtration.

Flocculation with acid:

cc. 0·1 *N* HCl added to 50 cc. of the haemolysed blood	7	8	9	9
p_H (quinhydrone)	5·86	—	5·27	5·30
mg. nitrogen per 3 cc. after removal of the precipitate	21·10	20·80	20·39	20·59
	21·25	20·84	20·44	20·49
Av.	21·18	20·82	20·42	20·54
mg. nitrogen per 3 cc. calculated on the original solution	24·15	24·17	24·10	24·24
Loss of nitrogen in % of total N	4·13	4·05	4·33	3·77

Exp. 2. This experiment was performed on human blood in the same way as in Exp. 1. The amount of nitrogen removed by filtration was 2·37 % of the total N content. On adding acid to the haemolysed blood the following percentages of nitrogen were removed with the precipitate:

At p_H	6·39	6·20	5·72	4·98
%	4·02	4·57	4·64	3·87

Exp. 3. On adding acid to haemolysed horse blood as described in Exp. 1, 4·41 and 4·31 % of the total N were removed.

To 100 cc. of the haemolysed well washed blood corpuscles 7 cc. of anti-horse immune serum from the goat were added and the precipitate was

removed by centrifuging in stoppered tubes. The nitrogen content of the supernatant liquid was determined. After correction for the dilution and the nitrogen content of the immune serum it was found that 4·31 % of the original amount of nitrogen had been removed with the precipitate.

To the solution of the haemolysed corpuscles in this experiment sodium chloride was added to 0·9 %. After centrifuging in stoppered test-tubes nitrogen was determined on the clear liquid. 7·92 % of the total N had been removed with the precipitate. On adding immune serum and sodium chloride at the same time to the haemolysed blood 11·33 % of the nitrogen content was removed, which corresponds to the summarised effects of the immune serum and of the sodium chloride alone. The effect of sodium chloride is taken as an expression of a "reversible haemolysis" in the sense of Brinkman and Szent-Györgyi [1923].

A series of experiments of this kind was made with human, horse and cow bloods. The results were very often less convincing than in the experiments quoted above. Sometimes the same percentage of nitrogen was removed by filtration through a Seitz filter as by precipitation with acid. In the two experiments given here however more nitrogen was flocculated out by means of acid (4–4·6 %) than was removed by filtration (2·4–2·8 %). In other cases there was never a full agreement between the results obtained after adding immune serum or acid to the haemolysed blood. The figures obtained with acid in one and the same blood also varied considerably, owing to circumstances which could not be explained.

It seems probable that the stroma protein in normal human and horse blood should amount to not more than 4–4·5 % of the total protein content of the erythrocytes. Furthermore the experiments tend to show that the stroma protein obtained by haemolysis of human or horse blood with 5 volumes of distilled water flocculates quantitatively on adding acid to the liquid to a p_H of about 5·5.

Discussion.

The stroma protein has a characteristic composition differing from that of the other blood-proteins. Its physical properties also are very characteristic. Its voluminous consistency, its elasticity and shrinking tendency resemble those of fibrin. Owing to its acid nature it certainly contributes to the negative electric charge of the membrane as is shown by its de-ionisation and flocculation on the addition of acid to a p_H of about 5·5. Concerning its colloidal structure it is difficult to make any statements.

Together with the lipoids it forms the membrane which according to Weidenreich [1903], Gough [1924] and others surrounds the haemoglobin sol. The lipoids are found by different authors to be slightly more than 1 % of the dry substance of the erythrocytes. If the stroma protein is about 4 % of the dry substance, as suggested above, then the lipoids are about 20 % of the dry substances of the membrane. The same order of magnitude 16–24 %

was also found by Beumer and Bürger [1912], who showed that the lipoids are to be found quantitatively in the membrane of the erythrocytes. The view that the stroma protein together with the lipoids form a definite structural unity is furthermore supported by the recent findings of Iwatzuru [1926], Grigant, Debray and Furstner [1925] and Vahlquist [1931] that the erythrocytes have a definite content of lipoids in spite of the changes occurring in serum in connection with the resorption of food.

As to the question of the occurrence of a basic protein in the erythrocyte membrane it will be very difficult to get any direct evidence supporting such a view. It is questionable whether there are other proteins in the membrane than that analysed here.

The fact remains that the erythrocyte membrane is non-permeable to cations. Possibly the haemoglobin, even although its isoelectric point is on the acid side of the p_H of the blood exerts a certain influence in the direction supposed by Michaelis and Mond or that globin with its isoelectric point at p_H 7·5 [Roché, 1930] acts in the same way. Taking into account the strongly acid character of the stroma protein it must however be admitted that the permeability or non-permeability of the erythrocyte membrane to ions is still far from being fully explained.

On the other hand, seeing that the acid proteins have strong antigenic properties, the acid characters of the stroma protein would be consistent with the suggestion that some of the immunising properties of the red blood corpuscles were located in it.

Methods.

The determination of tyrosine and tryptophan.

Amongst the methods recommended for the determination of tyrosine, that of Folin and Ciocalteau [1927], as modified by Folin and Marenzi [1929], is probably the most reliable. For reasons which will be stated later, however, some alterations of their technique can be made with advantage. Thus, the method can be very much simplified if the silicic acid, dissolved by alkali from the test-tubes during hydrolysis, is not filtered off. It will be removed together with the tryptophan-mercury compound, and does not interfere with the tryptophan analysis. It is not even necessary to remove the brownish colour by means of kaolin if the readings are made in a photometer. A second filtration is thus avoided, and the time for which the sulphuric acid acts upon the tryptophan is shortened. In order to avoid humin formation, the alkaline solution can furthermore be diluted by 6 cc. water before 3 cc. 7*N* H_2SO_4 are added. These alterations are not essential.

A more important question is that of the turbidity which tends to appear after boiling the tyrosine solution containing mercuric sulphate and sulphuric acid. Folin and Ciocalteau recommended boiling for 15 minutes, but, in order to avoid any turbidity, Folin and Marenzi shortened the time to 5 minutes. This however is not sufficient. The colour developing after the addition of

nitrite will not be maximum after such brief boiling, and the agreement between the figures is not nearly so good as when the boiling is continued for 10 or 15 minutes. In the former case, a difference of 4–10 % between the duplicates was often obtained, while in the latter the difference seldom exceeded 1 %. The longer time of boiling is therefore preferable. The turbidity which appears can be filtered off before adding the nitrite solution. After boiling, the flasks are cooled and left standing for several hours. Water is then added to 100 cc. and, after mixing, the solution is filtered into another 100 cc. flask. To this, 1 cc. of the 2 % nitrite solution is added. The error thus introduced in the volume is negligible if the controls are analysed in the same way.

The Folin-Marenzi procedure is to be corrected at least in one detail. They state that the reading should be made "without undue delay." By measuring the colour intensity in the Zeiss photometer, it will be found that the colour of the Millon reaction does not reach its maximum until 8–10 minutes after adding the nitrite solution. After 3 minutes, the colour intensity is 88 % of the final, after 4 minutes 95 % and after 5 minutes 97 %. Since fading sets in 20–25 minutes after the addition of the nitrite, the reading is to be made from 10 to 18 minutes. The reading is made with filtrum S. 50 of the photometer.

In spite of Folin and Ciocalteau's statements to the contrary, no true proportion could be found between the amount of tyrosine present and the intensity of the colour.

This is evident from the following table which contains the results of duplicate analyses of three different concentrations of pure tyrosine.

Amount of tyrosine in 100 cc., mg.	0·5	1·0	2·0
Scale readings (S. 50; $d=2{\cdot}006$)	65·1	44·5	20·8
	65·1	44·2	21·2
ϵ or $-\log_{10}\frac{I_p}{I_a}$	0·186	0·351	0·682
	0·186	0·354	0·674
ϵ for 1 mg. tyrosine ($d=2{\cdot}00$)	0·372	0·351	0·341
	0·372	0·354	0·337

This reaction can be used for colorimetric purposes, only if the standard approaches the unknown very nearly.

For the photometer, a table is made, in which the non-logarithmic abscissa expresses the amounts of tyrosine used, and the numbers on the scale are given on the logarithmic ordinates. The points fall very nearly on a straight line.

The accuracy of the method was checked by subjecting known amounts of tyrosine (0·5–2·5 mg.) to hydrolysis together with 70 mg. of gelatin. From 91 to 102 % were recovered, the mean figure being 95·8 ± 0·94 % (17 analyses).

The determination of tryptophan did not give nearly such good results as that of tyrosine. When pure tryptophan mixed with tyrosine and gelatin was subjected to hydrolysis and subsequent analysis, according to the micro-method of Folin and Marenzi, only 71–98 % of the calculated amount of

tryptophan was recovered. The mean figure was 82·4 ± 2·18 % (17 analyses). The greatest accuracy in the duplicates was obtained by using pure tryptophan solutions directly, without precipitating with mercuric sulphate. However, the true proportionality in colour which Folin and his co-workers found, was not obtained even here. Thus, using tyrosine as standard, the following figures were obtained for ϵ, re-calculated for 1 mg. tyrosine in 100 cc. (S. 72).

When	0·2	mg. tyrosine	was taken in 100 cc.	0·935
,,	0·4	,,	,,	0·873
,,	0·8	,,	,,	0·832
,,	1·5	,,	,,	0·783
,,	2·0	,,	,,	0·754

By plotting the figures for the amounts of tryptophan (or tyrosine) on semilogarithmic paper, as stated above, a straight line was obtained, and the reaction is thus to be considered as quite reliable for pure tryptophan solutions. The cause of the difficulties met with in the ordinary analysis, might have been incomplete precipitation of tryptophan, or an interference of mercury salts with the colour reaction. The latter seems to be the more probable since it was found that even small amounts of mercuric salts, added to pure tryptophan solutions, impaired the results, even if the boiling with hydrochloric acid were performed in the usual way. This uncertainty could not be avoided and therefore a correction for the loss was made in calculating the tryptophan content.

By the technique used the figures found for the tyrosine and tryptophan contents of oxyhaemoglobin are about the same as found by Folin and Ciocalteau, *viz.* 2·84 % tyrosine and 1·26 % tryptophan against 3·15 and 1·28 % as found by these workers.

The determination of iron.

The volumetric method with permanganate as elaborated by Murray [1924] was found to give good results down to 1 or possibly 0·5 mg. Fe. It was necessary however to determine smaller quantities. For that purpose the method recommended by Lorber [1927] was tried. After some difficulties with the ashing of the material reproducible results were obtained. The intensity of the yellow colour developed in the alkaline solution containing ferric iron and sulphosalicylic acid was measured in the Zeiss photometer in filter S. 47. No true proportionality between the amount of iron present and the colour intensity was obtained, the smaller amount giving the relatively stronger colour. On plotting the figures found with solutions of ferric chloride, or ferric alum on semilogarithmic paper as described above, the points, however, fall nearly on a straight line. Hence such a table is used in the ordinary work. The colour is very stable and is not influenced by moderate changes in the concentration of ammonia or salts.

The following procedure was found satisfactory. The substance was ashed in a 1·5 × 3 cm. platinum dish in an electric oven for an hour or two. Potassium nitrate was then added (about 50 mg.) and the dish heated in the oven for 10 minutes. After cooling 0·5 cc. concentrated sulphuric acid was added and the dish heated over a free flame. The contents of the dish were then transferred to a 25 cc. flask with 15 cc. of water. After cooling 20 % KOH solution was added and 0·5 cc. of a 20 % solution of sulphosalicylic acid. More of the KOH solution was added until the colour became violet. The solution was neutralised with a few drops of 20 % ammonia solution and then 0·5 cc. more of the ammonia solution was added. After dilution to 25 cc. and filtering, if the solution were not quite clear, the reading was made, preferably in filter S. 47.

0·02–0·05 mg. Fe was found a convenient quantity for a single analysis.

The accuracy obtained was not particularly good. Often single analyses fall out of the series. Generally there was a deviation of 2–3 % in the duplicates and an error of 5 % was frequent. For the purpose of this investigation however the method is to be considered as satisfactory provided a sufficient number of analyses be made.

Summary.

In order to study the protein of the erythrocyte membrane or stroma, membranes were isolated from horse and sheep bloods. After haemolysis with distilled water the "ghosts" were flocculated by adjusting the reaction with hydrochloric acid to a p_H of about 5·5. They were washed relatively free of haemoglobin with distilled water and a slightly acid dilute phosphate solution.

The membrane preparations contained 14–30 % haemoglobin according to the iron analyses.

The low nitrogen content, 14–15 %, and the nitrogen distribution according to Van Slyke correspond to those of the acid proteins. Herein and through its content of arginine and histidine the membrane protein differs from haemoglobin and globin. The figures for arginine and histidine found were 5·80 and 2·63 %, whereas those of haemoglobin are 3·32 and 7·75 %.

The membrane protein does not show any resemblance to fibrin or globulin. It differs from these through its content of tyrosine and tryptophan. Hence the membrane protein, if homogeneous, is a protein *sui generis*. The name stromatin is suggested for it.

The protein of the membrane or stroma amounts to roughly 4 % of the total protein content of the normal erythrocytes.

The bearing of these findings upon the permeability of the erythrocyte membrane towards ions is discussed.

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CLXXVII. THE USE OF THE SAKAGUCHI REACTION FOR THE QUANTITATIVE DETERMINATION OF ARGININE.

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The determination of this amino-acid has hitherto been based on the formation of urea or ammonia from it, either by boiling with 20 % potassium hydroxide solution, or under the influence of arginase. The first qualitative test for arginine, the diacetyl reaction of Harden and Norris [1911], has only recently been applied to its colorimetric estimation [Lang, 1932]. Sakaguchi's reaction for arginine [1925] is not only specific and very sensitive, but is also applicable to quantitative analysis. As the determination of arginine by Van Slyke's method demands a considerable amount of material, colour tests for small quantities of arginine would be very useful. As shown by Weber [1930], the Sakaguchi reaction can be used for the quantitative determination of 0·05–0·005 mg. arginine. Several precautions must however be taken.

In analysing some blood-proteins by the Van Slyke procedure, we used this reaction and found it reliable. The instructions given by Weber were at first followed. Instead of a colorimeter, a Zeiss step-photometer was used. This made a more accurate study of the colour development possible, and some valuable information was gained.

The procedure recommended by Weber was as follows. To 5 cc. of a solution containing 0·05–0·005 mg. arginine, 1 cc. 10 % sodium hydroxide solution was added, and 1 cc. of a 0·02 % solution of α-naphthol. After cooling on ice, 0·2 cc. of a hypobromite solution was added, and 4–6 seconds later, 1 cc. of a 40 % urea solution. The colorimetric reading should be made within 5 minutes. In the presence of α-naphthol and hypobromite a reddish colour is given by arginine and other mono-substituted guanidines. The colour is not given by any other amino-acids.

The colour is very sensitive to an excess of hypobromite, and therefore as soon as it has developed, urea is added in order to destroy the excess of hypobromite. Furthermore, as pointed out by Weber, the maximum colour is obtained with a certain amount of hypobromite only. This is to be considered in preparing the hypobromite solution and in performing the test on solutions which react with hypobromite. According to Weber the optimum

amount of hypobromite to be added is to be ascertained in each individual case. The hypobromite solution is prepared by dissolving 2 g. of bromine in 100 cc. of 5 % cooled sodium hydroxide solution. More or less bromine (3·5 or 1·6 g. bromine in 100 cc.) gives a fainter coloration.

The colour is greatly influenced by the temperature and if the reagents are not cooled, a much fainter colour is obtained. The test-tubes containing the mixed solutions are therefore kept for at least an hour plunged in ice before the addition of the hypobromite. The urea solution is cooled in the same way.

It takes about 10 seconds for the colour development to reach its maximum and the urea should not be added until this has been attained. Thus, in two series, a 12 % higher colour intensity was obtained when the urea solution was added after 10 seconds instead of after 5, as recommended by Weber. After reaching its maximum, the colour begins to fade about 20 seconds after the hypobromite solution has been added. The cooled urea solution is therefore to be added exactly 15 seconds after the hypobromite. Once stabilised, the colour remains constant for 6–7 minutes. This is important for the photometric reading. If the urea solution has not been cooled, the colour begins to fade in 4–5 minutes.

Owing to the lability of the colour, quite the same accuracy cannot be obtained by this reaction as by the diazo-reaction for histidine. The colour is not proportional to the amount of arginine present in the tests. With smaller amounts of arginine, a relatively greater colour intensity is obtained. Thus *e.g.* the coefficient of extinction $\epsilon\left(-\log_{10}\frac{I_x}{I_a}\right)$ calculated for 0·1 mg. arginine (d being 1 cm.), was 1·73 with 0·04 mg. arginine and 1·63 with 0·07 mg. The colour can thus be used for colorimetric purposes only if the standard is very close to the unknown. As this relative increase in colour intensity with decreasing amounts of substance is quite regular, a photometer can very well be used. The photometer scale is plotted on semilogarithmic paper. The amounts of arginine taken in a standard series are used as abscissae and the photometer scale as the logarithmic ordinates. The points fall on a straight line, and thus no further calculation is required. If the cooling is not sufficient, another line may be obtained. It is advisable each time to have controls of different concentrations in order to check this.

The solutions used are those of Weber.

1. An arginine solution containing 0·04–0·07 mg. arginine in 5 cc.
2. A 10 % solution of sodium hydroxide *e natrio*.
3. A 0·02 % solution of α-naphthol, made fresh each time by the dilution of 20 cc. of a stock solution (0·1 % in alcohol) to 100 cc. with water.
4. A hypobromite solution made by dissolving 2·5 g. (0·46 cc.) bromine in 100 cc. 5 % cooled sodium hydroxide solution. This solution keeps for months in the cold.
5. A 40 % urea solution.

Procedure. A test-tube, 150 × 18 mm., with 5 cc. arginine solution is cooled on ice. 1 cc. of sol. 2 (sodium hydroxide solution) is added and 1 cc. of sol. 3 (α-naphthol), the solutions mixed and the test-tube well embedded in ice. After a time (1 hour) 0·2 cc. of sol. 4 (hypobromite) is added with vigorous shaking, and exactly 15 seconds later 1 cc. of sol. 4 (cooled urea solution). The photometric reading is made against water in S. 50 within 6–8 minutes. For amounts of arginine between 0·04 and 0·08 mg., which is the range found most convenient in our experiments, a 0·5 cm. dish is used. The amount of arginine present is found directly from the curve on semi-logarithmic paper.

If all precautions are strictly taken, this method is as good as any ordinary colorimetric procedure. The duplicates agree to within 1–2 %. As neutralised phosphotungstic acid up to 1 % of the final arginine solution does not influence the colour intensity this colorimetric method may replace the alkaline hydrolysis of the Van Slyke procedure.

In analysing the basic fraction of the amino-acids of haemoglobin after dissolving the phosphotungstic precipitate in alkali, for histidine with the diazo-reaction of Pauly and for arginine with this method, we found that the figures agreed exactly with those found by Vickery and Leavenworth with their isolation method. (See the preceding paper.)

Summary.

The reaction of Sakaguchi with α-naphthol and hypobromite has been used for the determination of arginine in protein hydrolysates. The details of the colorimetric procedure have been studied. If the necessary precautions are taken, the reaction can be used for quantitative purposes. It is also applicable to the determination of the arginine content of the basic fraction of the amino-acids in the Van Slyke procedure.

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CLXXVIII. THE COLORIMETRIC DETERMINATION OF HISTIDINE.

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The reaction of iminazoles with diazobenzene sulphonic acid, which was introduced into protein chemistry by Pauly [1904], was used for an approximate quantitative estimation of histidine for the first time by Weiss and Ssobolew [1914]. Later Koessler and Hanke [1919] elaborated their colorimetric method for the determination of histidine in protein hydrolysates. The diazo-compound formed was also determined by volumetric titration by Lautenschläger [1918]. The compound was reduced by titanous chloride, and the excess of titanous salt was oxidised by ferric alum, thiocyanate being used as indicator.

Weiss and Ssobolew were well aware of the difficulties involved in the use of the reaction for quantitative purposes owing to the instability of the colour produced. They gave the following directions for the performance of the test. To 10 cc. of a histidine solution, containing 0·1 mg. histidine per cc., 1·5 cc. of the solution of the diazonium salt and then 3 cc. of a 10 % sodium carbonate solution should be added, thus assuring an optimum of purity and stability of colour.

Nevertheless Koessler and Hanke on theoretical grounds recommended a different procedure, which was also adopted by Hunter [1925] for the determination of iminazoles in the urine. They considered the reaction between the amino-group in histidine and the nitrous acid in the acid diazonium solution as undesirable. They therefore first mixed the diazonium salt with the carbonate solution and then after one minute added the histidine solution. Another reason in favour of this procedure was that the concentration of diazotate became maximum before the addition of the histidine solution. It is very probable according to them that the compound entering into reaction with histidine is a diazohydrate, $NaOSO_2C_6H_4N{=}NOH$ or an isomeric nitrosamine $NaOSO_2C_6H_4\underset{\displaystyle NO}{\overset{\displaystyle |}{N}}H$, which is formed through hydrolysis of the diazotate. By carefully noting the time for the addition of the histidine solution and the colorimetric reading it should be possible to obtain close agreement between the histidine content and the colour produced. Hanke

and Koessler [1920] used their method for the determination of the histidine content of protein hydrolysates. The histidine is of course to be separated by phosphotungstic acid or by the Kossel-Patten procedure from tyrosine, which also gives the reaction.

In analysing some blood-proteins for their content of histidine by the Koessler and Hanke method, using the Zeiss step-photometer, I have found it difficult to obtain reproducible results with their technique. With the photometer the development and fading of the colour could be followed much more easily than with a colorimeter, and furthermore the optimum field of the spectrum could be selected. Immediately after mixing the reagents a yellow colour appears which is less suitable for reading than the reddish colour which develops in the course of 2–3 minutes.

On attempting to determine the degree of light absorption, using solutions containing 0·08–0·01 mg. *l*-histidine dihydrochloride per cc., no definite coefficient of extinction was obtained. The readings were made with filter S. 50 where the reddish colour has its maximum of absorption. According to the descriptions given by Koessler and Hanke the histidine solution was added exactly one minute after mixing the other two solutions. The intensity of the colour should be maximum after 6 minutes and remain at this level for 2–3 minutes. However, the colour was found to be maximum in 3–3½ minutes after addition of the histidine solution and fading could be observed 5–6 minutes after mixing. Furthermore it was indifferent whether the histidine solution was added immediately after the diazo-solution or 2 minutes later. The coefficient of extinction, ϵ or $-\log_{10}\frac{I_p}{I_a}$, d being 1 cm., varied between 2·09 and 2·31 as calculated for an amount of 0·1 mg. histidine in 8 cc. of the final reaction mixture. The mean figure for 32 determinations was $2{\cdot}19 \pm 0{\cdot}0140$. The error of the reading is very small and does not influence the results. In spite of its very common use during the last few years the reaction is apparently too labile to be considered reliable.

Without making any statements about the possible cause of this lability in the reaction it is possible to trace the influence of the diazotate solution. If the transformation of diazonium salt to diazotate, *i.e.* the alkalisation of the diazonium solution, proceeds in the presence of histidine a much better agreement between histidine content and colour intensity is obtained. When the diazonium solution was added last, the corresponding figure for ϵ was $2{\cdot}16 \pm 0{\cdot}0015$ (mean of 32 determinations). A still closer agreement was obtained when the original procedure of Weiss and Ssobolew was followed and the carbonate solution added last even when the reagents of Koessler and Hanke were used. With this technique it was observed that the absorption of light increased with prolongation of the interval before the carbonate solution was added. If 20 seconds elapsed between the admixture of the diazonium and the carbonate solutions, the figure for ϵ (calculated as stated above) was found to be 2·34; if 10 minutes 2·46, and if 20 minutes 2·51. After

an hour no further increase was obtained. The same values were obtained even when the specimens had been standing with the diazonium solution for 4 hours before its alkalisation. The final figure for ϵ found was $2{\cdot}66 \pm 0{\cdot}0054$ (mean of 23 determinations).

The colour reached its maximum of intensity in $3\frac{1}{2}$–4 minutes and fading began not less than 8 minutes after adding the carbonate solution.

With the technique given below, this reaction seems to be the most reliable of all the colorimetric reactions for amino-acids used for quantitative purposes, due regard being given of course to its non-specificity in the presence of tyrosine, guanine, adenine, phenols, iminazoles, sulphides and ammonium salts.

Whatever the explanation of this difference in the colour development may be, it seems probable that a reaction proceeds between histidine and diazobenzenesulphonic acid even in a slightly acid solution, as is evident from the increasing colour intensity with an increasing interval before alkalisation. It seems probable that this reaction does not consist only in a deamination of histidine in the presence of nitrous acid but that the diazo-compound is coupled with the iminazole nucleus.

At least the conditions for a stoichiometric course of the colour reaction are more favourable here than in the alkaline diazotate solution in which apparently several undesirable reactions take place. The diazotate solution itself acquires a yellow tint in half an hour which indicates the occurrence of such changes.

As pointed out by Weiss and Ssobolew the intensity of the colour is largely influenced by changes in the reacting solutions. The maximum of colour intensity is not obtained with the reagents recommended by Koessler and Hanke, but these nevertheless give the most easily reproducible figures. If a 10 % carbonate solution is used instead of 1·1 %, no colour at all is obtained, but if the stronger carbonate solution is used, together with a fivefold stronger diazonium solution, the colour obtained is 40 % stronger than with the ordinary reagents.

Technique of the reaction.

Solutions.

(1) A neutral or faintly acid histidine solution containing 0·05–0·005 mg. histidine per cc.

(2) A diazonium solution prepared according to the descriptions of Koessler and Hanke. To 1·5 cc. of a solution containing 0·9 g. sulphanilic acid and 9 cc. conc. HCl in 100 cc. are added 1·5 cc. of a 5 % sodium nitrite solution. The mixture is cooled on ice for 5 minutes. Then 6 cc. of the nitrite solution are added with shaking, and after cooling for 6 minutes water is added to 50 cc. The diazonium solution should be kept on ice. It keeps for 24 hours. The solution of sulphanilic acid keeps for years. The best results are obtained if the 5 % solution of sodium nitrite is freshly prepared.

(3) 1·1 % sodium carbonate solution (Kahlbaum's anhydrous sodium carbonate).

To 1 cc. of solution 1 (histidine) 2 cc. of solution 2 (diazonium) are added. After 1–3 hours 5 cc. of solution 3 (carbonate) are added. The reading is made with filter S. 50 of the step-photometer 4–8 minutes after the addition of solution 3.

The amount of histidine present in the specimen is calculated from the figure found for ϵ or $-\log_{10}\frac{I_p}{I_a}$. If 1 cc. solution or 8 cc. reaction mixture contains 0·1 mg. histidine the figure for $-\log_{10}\frac{I_p}{I_a}$ is 2·66, d being 1 cm. If the tests are performed as described above the colour intensity is directly proportional to the histidine content when this is from 0·05 to 0·005 mg. per cc. Almost the same figure for ϵ was obtained on three different occasions with a 3 to 6 months' interval, *viz.* 2·66, 2·69 and 2·66. These figures were obtained with a specimen of *l*-histidine dihydrochloride, which contained 18·57, 18·41 % N (Kjeldahl), calc. 18·43 %.

Influence of foreign substances.

It is known that the development of the colour is prevented by alkalis or acids as well as in the presence of heavy metal salts. If the histidine is dissolved in 1 cc. of 0·1 *N* NaOH no colour is obtained. The solution must not have an alkalinity stronger than 0·01–0·001 *N*. The acidity must not exceed 0·01 *N*. The presence of 0·01 mg. $HgCl_2$ impaired the colour. The same applies to the copper and silver salts. Small amounts of ferric salts on the other hand do not inhibit the reaction, thus 1 mg. $FeCl_3$ does not influence the test. A moderate concentration of sodium chloride has no influence.

Owing to the necessity of separating the basic amino-acids the influence of phosphotungstic acid is important. Fortunately the colour intensity is not affected, if the histidine is dissolved in a 1–0·5 % solution of phospho-24-tungstic acid (Wu). When the concentration is increased to 3 % a slight impairment is apparent.

There are apparently no disadvantages in using this method for the determination of histidine after dissolving the basic fraction of the amino-acids with alkali in the ordinary Van Slyke procedure. In previous work [1932] the histidine content of oxyhaemoglobin was determined in this way in the basic fraction of the amino-acids. The figures found corresponded closely to those given by Vickery and Leavenworth [1928].

Summary.

The colorimetric determination of histidine by the Pauly reaction has been studied. By means of the Zeiss photometer the development and the stability of the colour were followed. It was found that the procedure, recommended by Hanke and Koessler, which has been largely applied during recent years, gives very unsatisfactory results. Using their reagents, however, it is possible by changing the technique to get easily reproducible figures.

A true proportionality is obtained between the intensity of the colour and the amount of histidine present in the range 0·05–0·005 mg. histidine per cc. The colorimetric method, thus modified, is recommended for the determination of histidine in the basic fraction of the amino-acids in the protein analysis according to Van Slyke.

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CLXXIX. INVESTIGATIONS IN ENZYME ACTION DIRECTED TOWARDS THE STUDY OF THE BIOCHEMISTRY OF CANCER.

THE ACTIVATION OF PANCREATIC PRO-LIPASE.

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The marked increase in the lipolytic activity of extracts of fresh pancreas towards olive oil emulsion and similar substrates which is occasioned by the presence of a small proportion of blood-serum has been demonstrated by many workers. Differences in the augmenting effects of human sera have been utilised for diagnosing such abnormalities as the presence of malignant disease. The phenomenon of lipolytic augmentation was studied in particular by Rosenheim [1910; 1915; 1925] and by Shaw-Mackenzie [1911; 1912; 1913; 1914; 1931], who concluded that in many instances serum from patients suffering from progressive cancer activated the enzyme to a less extent than normal serum. More recently Corran and Lewis [1928] found that their experiments confirmed these findings.

Some preliminary work by Sanders at this laboratory [1932] also suggested that the augmenting effect of human serum was diminished in cancer and was subsequently raised by lead or radium treatment, although the change was not sufficiently regular for following the progress of the disease. On the other hand the augmenting effect of the serum of tumour-bearing mice appeared to be slightly raised.

The present paper describes experiments devised to enquire into certain problems connected with the mechanism of lipolytic activation and with the effects thereon of some organic and inorganic compounds of therapeutic interest, together with an examination of the activity of normal and cancerous blood-sera and other physiological fluids on a modified form of the enzyme material.

The increased activity of the enzyme in presence of serum is ascribed to the interaction of a thermostable co-enzyme in the blood. Corran [1929] showed that the agent is particularly associated with the albumin and pseudoglobulin of the serum and is absent from the salt and lipin fractions. A co-enzyme with similar activating properties is present in glycerol extracts of pancreas and its removal results in the complete inactivation of the preparation.

The production of such inactive pro-lipase was described by Rosenheim [1910] who showed it to be thermolabile and capable of being activated by tissue extracts and serum. The separation of the pancreatic pro-lipase and co-enzyme was effected by filtration or by precipitation of the pro-lipase by diluting the extract with 4 parts of water and separating the flocculent precipitate. This could be subsequently dried with alcohol and ether and used in the form of powder. Magnus [1904] had also separated the activator of lipolytic liver extract by dialysis.

In the present research a form of this pro-enzyme has been very extensively employed. The values for the activating effects of sera and other substances have been determined with this material, as it was thought that it would be an advance in the technique of the lipase test for cancer diagnosis to measure the activating power of serum on the inactive pro-lipase in the absence of the pancreatic co-enzyme. In this way the full augmenting, or, as it is preferably termed, the "activating power" of the serum is measured and the results should therefore be more definite and reliable.

EXPERIMENTAL.

A. Methods.

1. *Technique of tests.* The tests were carried out in small (1 oz.) bottles fitted with rubber bungs. The lipase extracts were introduced by means of a micro-burette and the olive oil emulsion by means of a wide-bored pipette. The reagents were always added in the order (1) lipase extract, (2) serum, co-enzyme or other activator, (3) water, (4) olive oil emulsion, since Corran [1929] had found that the augmenting power of the serum was affected by the order in which the reagents were mixed, although in no case have we found the variation of lipolysis to be greater than could be ascribed to experimental error. Possibly the variations in the lipolysis found by previous workers were caused by the flocculation of the enzyme-protein complex on diluting the glycerol extract. This change, as shown in a later part of this paper, very readily occurs within certain limits of p_H. The mixtures were incubated for 48 hours at 37°, being vigorously shaken during the whole period in a special shaking machine which dealt with a total of 96 bottles in an incubator 12″ × 14″.

It had previously been determined that the interacting phases were in complete equilibrium after 48 hours' incubation under these conditions. The acid liberated (*i.e.* the degree of lipolysis) was determined by the usual method of titration to phenolphthalein with $N/10$ NaOH in presence of alcohol and ether.

2. *The enzyme material.* The following pancreatic extracts were employed in this work.

(*a*) Glycerol extracts; 1 part pig gland: 2 parts glycerol.

(*b*) Aqueous extracts; 1 part pig gland: 2 parts water.

(*c*) Glycerol and aqueous extracts diluted 10 times with distilled water.

(*a*) *Glycerol extracts.* These were prepared from the minced glands exactly as described by previous workers. With these extracts it was found that duplicate tests often showed marked variations. Centrifuging the extracts for 3 minutes at 1500 r.p.m. somewhat improved the consistency of the results.

(*b*) *Aqueous extracts.* Much more consistent results were obtained by employing extracts prepared by shaking the minced gland with 2 parts of sterile distilled water for 2 hours, filtering the milky liquid through a fine mesh sieve and centrifuging for 3 minutes at 1500 r.p.m.

Contrary to the experience of some workers, we found that this extract, if prepared with aseptic precautions, kept cool (in refrigerator at 3–5°) and further preserved from bacterial action by the addition of a few drops of chloroform to each 100 cc., did not lose its activity for a period of many weeks.

Exp. 1. Stability of aqueous pancreatic extract.

0·5 cc. extract incubated for 48 hours with 4·0 cc. standard olive oil emulsion, prepared as described in section 3.

Age of extract	Acid liberated (cc. *N*/10 NaOH)
24 hours	31·8
3 weeks	31·4
8 „	30·0
10 „	25·2

(*c*) *Pro-lipase extract.* We were able to repeat the separation of the inactive pro-lipase from the glycerol extract of pancreas by Rosenheim's dilution technique and to confirm that it was reactivated by serum obtained from man and animals and also by tissue extracts including boiled pancreas extract[1].

Exp. 2. Test of pro-lipase as prepared by Rosenheim's method.

Glycerol extract of minced pig's pancreas 1 part gland: 2 parts glycerol; kept 48 hours; filtered through double muslin; 10 cc. run into 40 cc. distilled water in tall cylinder; mixed gently; kept 20 hours; deposit centrifuged; washed twice with 20 cc. water; made up to 10 cc. with 80 % glycerol.

Pro-lipase cc.	Activator cc.	Olive oil emulsion cc.	Titration cc. *N*/10 NaOH
0·5	—	5·0	1·2
0·5	1·0 co-enzyme*	5·0	19·6
0·5	0·5 ox serum	5·0	32·7
0·5	0·5 rabbit serum	5·0	25·6
0·5	0·5 human serum	5·0	30·8
—	1·0 co-enzyme*	5·0	0·85
—	0·5 ox serum	5·0	0·60

* Prepared by boiling equal volumes of aqueous pancreas extract and water and filtering.

Unfortunately we found much difficulty in obtaining sufficient quantities of the precipitate by this method. By the following slight modification, however, a definite and speedy flocculation was invariably obtained.

90 cc. of distilled water in a 100 cc. cylinder were acidified with exactly 0·1 cc. of pure lactic acid and 10 cc. of the aqueous extract were added. In about 6 hours the flocculated pro-lipase formed a compact sediment in the lower 10 cc., the supernatant liquid usually being quite clear. This was siphoned

[1] I am indebted to Dr Rosenheim for a private communication regarding his method.

away, the deposit of pro-lipase centrifuged and treated as described above. This simple technique was evolved after many unsuccessful efforts to procure a suitable pro-lipase by other methods, including alcohol or acetone precipitation, dialysis, precipitation by magnesium sulphate, *etc.*

The flocculated pro-lipase when washed and made up to a suitable volume was quite inactive towards olive oil emulsion but became definitely active in the presence of serum or of co-enzyme solutions prepared from glycerol or aqueous pancreas extracts by diluting them with an equal volume of water, boiling and filtering the coagulum from the clear fluid. If so desired, the precipitated pro-lipase could be washed with acetone and ether and thus obtained as a dry powder without appreciable loss of potency.

Exp. 3. Acid-flocculated pro-lipase.

Pro-lipase	Activator cc.	Olive oil emulsion cc.	Titration cc. *N*/10 NaOH
0·5 cc. aqueous emulsion	—	4·0	2·0
0·5 „	1·0 co-enzyme	4·0	28·5
0·5 „	0·5 ox serum	4·0	35·3
approx. 0·5 mg. powder	—	4·0	1·4
„ 0·5 „	1·0 co-enzyme	4·0	25·0
„ 0·5 „	0·5 ox serum	4·0	28·3

(*d*) *Diluted extracts.* Preliminary experiments carried out to determine the optimum quantities of the reagents for the lipase tests demonstrated that when much smaller quantities of the ordinary lipase extract than hitherto utilised were employed in conjunction with the usual volume of olive oil, a considerable amount of lipolysis still ensued. Thus, when only 0·05 cc. of either glycerol or aqueous extract was used in conjunction with 0·5 cc. of ox serum the titration obtained was approximately the same as when the full 0·5 cc. was used.

Exp. 4. Effect of diminishing amount of lipase extract.

Lipase cc.	Activator cc.	Olive oil emulsion cc.	Titration cc. *N*/10 NaOH
0·5 aqueous	—	4·0	40·7
0·1 „	—	4·0	19·3
0·05 „	—	4·0	1·8
0·05 „	0·5 ox serum	4·0	41·8
0·5 glycerol	—	4·0	35·2
0·05 „	—	4·0	2·0
0·05 „	0·5 ox serum	4·0	33·5

It will be noted that the extent of lipolysis does not vary directly with the quantity of lipase added; below a critical minimum amount the reaction is practically zero. This suggests that a certain concentration on the surface of the droplets of oil is needed to enable the reaction to proceed; the addition of co-enzyme may enable the orientation of the colloidal particles to take place with small amounts of the actual pro-enzyme material. In a similar fashion Willstätter [see Waldschmidt-Leitz, 1929, p. 65] has suggested that the effect of proteins and salts such as calcium oleate in augmenting lipase action is

dependent upon the formation of complexes which bring the enzyme into more intimate contact with the substrate.

Experiments with diluted glycerol and aqueous lipase were then made which showed that 0·5 cc. of either extract, previously diluted 10 times with glycerol or water respectively, was able, in the presence of 0·5 cc. of serum, to hydrolyse to the same extent as 0·5 cc. of the original extract.

Exp. 5. Comparison of undiluted and diluted extracts.

G. = Glycerol extract. A. = Aqueous extract.

Lipase diluted cc.	Lipase undiluted cc.	Activator cc.	Olive oil emulsion cc.	Titration cc. $N/10$ NaOH
—	0·5 G.	—	4·0	29·6
—	0·5 A.	—	4·0	25·7
0·5 G.	—	—	4·0	1·4
0·5 G.	—	1·0 co-enzyme	4·0	20·9
0·5 G.	—	0·5 ox serum	4·0	29·3
0·5 A.	—	—	4·0	0·95
0·5 A.	—	1·0 co-enzyme	4·0	17·3
0·5 A.	—	0·5 ox serum	4·0	26·6

This demonstrates that the glycerol and aqueous extracts contain a great excess of pro-enzyme over the activator, a fact which suggests that the part played by the tissue juices and the blood-serum in activating the inert enzyme material (in which form it largely exists in the gland), is of the utmost importance in fat metabolism in the body.

By diluting these solutions sufficiently the proportion of co-enzyme can be reduced to negligible amounts—the diluted enzyme solution will therefore be inactive but will contain a relatively large amount of latent pro-enzyme which can readily be activated by adding *e.g.* 1 cc. co-enzyme solution or 0·5 cc. serum, in which case titrations of 20–30 cc. $N/10$ NaOH are obtained after 48 hours' incubation with 4·0 cc. olive oil emulsion. It was observed that ox serum was invariably more effective in activating the dilute extracts than an equal volume of co-enzyme; 0·3 cc. of serum was usually able to activate to its full extent 0·05 cc. of undiluted extract or 0·5 cc. of diluted solution.

Exp. 6. Activation value of ox serum for diluted extracts.

Lipase diluted extract cc.	Activator cc.	Olive oil emulsion cc.	Titration cc. $N/10$ NaOH
0·5	—	4·0	1·2
0·5	0·1 ox serum	4·0	3·6
0·5	0·2 ,,	4·0	14·5
0·5	0·3 ,,	4·0	32·8
0·5	0·4 ,,	4·0	31·6
0·5	0·5 ,,	4·0	32·0
0·5	1·0 co-enzyme	4·0	18·0
0·5	—	—	—

It will be seen from these figures that the diluted extract can be utilised for certain experiments in which pro-lipase free from co-enzyme is required, thereby saving the time necessary for the isolation of the pro-enzyme by the flocculation methods.

The inactive material in both forms has been found very suitable for estimating the activation value of sera and other fluids; the figures obtained are a direct measure of the activity bestowed on the previously inert material.

The validity of this statement was proved by experiments in which varying quantities of pro-lipase were activated by a constant volume of serum. When the co-enzyme value of the serum was fully exerted it was not possible to cause a greater lipolysis by increasing the content of pro-lipase, *i.e.* the reaction was limited by the co-enzyme available.

Exp. 7.

Pro-lipase obtained by sedimentation from 20 cc. of aqueous extract of pancreas in presence of 0·1 % lactic acid; centrifuged and washed with water 3 times; volume made up to 20 cc. with distilled water. Action on olive oil emulsion tested after incubation for 48 hours at 37°.

Pro-lipase cc.	Serum cc.	Water cc.	Emulsion cc.	Titration cc. *N*/10 NaOH
0·5	—	1·5	4·0	1·1
1·0	—	1·0	4·0	1·4
0·25	0·5	1·75	4·0	37·8
0·5	0·5	1·5	4·0	38·8
0·75	0·5	1·25	4·0	38·0
1·0	0·5	1·0	4·0	36·4
0·5	0·2	1·8	4·0	30·3

3. *The olive oil emulsion.* The oil was purified with sodium hydroxide as recommended by previous workers. The sodium oleate was prepared by neutralising pure oleic acid. In the method used for routine tests an emulsion was prepared by adding 3·75 cc. of 7·5 % sodium oleate to 100 cc. of purified olive oil, instead of 25 cc. of 1 % as used by other workers. This gave a stable emulsion and reduced the quantity of aqueous phase to a minimum, a condition which was desired for some of the tests. Various experiments proved that this proportion of sodium oleate emulsified the oil suitably and gave a reliable substrate.

Exp. 8. Effect of sodium oleate.

Lipase (aqueous) cc.	Serum cc.	Water cc.	Emulsion cc.	Olive oil cc.	Titration cc. *N*/10 NaOH
0·5	—	1·0	—	4·0	28·6
0·5	—	1·0	4·0	—	36·1
0·5 (diluted by 10)	0·5	0·5	—	4·0	27·2
0·5 „	0·5	0·5	4·0	—	33·0

4. *Quantities of reagents.* For most of the preliminary work the following volumes of reacting fluids were found to be very suitable:

	cc.
Lipase extract	0·5
Serum	0·5
Co-enzyme or other activating solution	1·0
Olive oil emulsion	4·0
Water to bring total volume to	6·5

For quantitative tests on sera this method was afterwards modified by reducing the volume of serum to 0·2 cc. in accordance with the results of the

following experiment in which the activation values of varying quantities of ox serum towards the pro-lipase were determined.

Exp. 9. Activating values of varying amounts of ox serum towards pro-lipase.

1·0 cc. samples of pro-lipase, inactive towards olive oil emulsion, were distributed into a series of bottles; amounts of ox serum from 0·05 to 1·0 cc. added; incubated 48 hours at 37° with 4 cc. olive oil emulsion.

Serum (cc.)	Titration cc. *N*/10 NaOH
0·00	2·0
0·05	11·0
0·075	16·5
0·10	25·6
0·15	31·4
0·20	34·5
0·25	36·3
0·30	37·3
0·40	38·4
0·50	38·3
0·75	38·7
1·00	37·8

It will be observed that the addition of serum up to 0·3 cc. results in increasing values in the acid liberated, but beyond this, very little alteration in the lipolytic value is brought about.

This result was confirmed by several tests using different samples of pancreatic lipase and serum. The general character of the reaction is also indicated by Exp. 6.

It is to be concluded therefore that for comparison of the activation values, the addition of large volumes of serum is not indicated.

B. Results of experiments.

1. *Action of some colloidal substances on pro-lipase.*

As demonstrated above, sodium oleate has the power of augmenting the action of lipase solutions in presence or absence of serum and of diluted lipase extracts in the presence of serum. This action may be due to (1) possible activation of the pro-lipase, (2) stabilisation of the olive oil emulsion.

By testing with diluted lipase extracts and precipitated pro-lipase it was found that the augmenting action must be ascribed to the influence on the substrate. Such extracts, in presence of 1 cc. of 1 % sodium oleate, remained quite inactive. Many other organic and inorganic colloidal substances were also tested for possible power towards pro-lipase.

It was found that while 2 % gelatin, 1 % gum arabic, 1 % gum tragacanth, 1 % agar were able definitely to augment the action of lipase on olive oil, none was able to activate the pro-lipase. The following series of compounds was tested and found to have no such activating power: starch (1 % suspension, or 0·5 % solution of Lintner's soluble starch), caseinogen, haemoglobin from ox blood corpuscles, colloidal zinc sulphide, suspension of powdered charcoal.

On the other hand a solution of raw egg-white in 5 times its volume of distilled water and a solution of "colloidal iron" (as supplied for protein precipitation for MacLean's method of estimation of blood-sugar) had a distinct effect in activating the pro-lipase.

The results are set out in Table I.

Table I. *Action of colloids on pro-lipase.*

Lipase cc.	Augmentor cc.	Water cc.	Emulsion cc.	Titration cc. $N/10$ NaOH
0·5 aqueous dil.	—	0·5	4·0	1·4
0·5 ,,	1·0 gum arabic	0·5	4·0	1·3
0·5 ,,	1·0 egg-white	0·5	4·0	28·0
0·5 ,,	1·0 gum tragacanth	0·5	4·0	2·3
0·5 ,,	1·0 "colloidal iron"	0·5	4·0	29·0
0·5 ,,	1·0 egg-albumin	0·5	4·0	2·0
0·5 pro-lipase	1·0 egg-white	0·5	4·0	23·6
0·5 ,,	1·0 "colloidal iron"	0·5	4·0	10·0

It is not improbable that the activity of the egg-white was due to some unknown substance adsorbed on the proteins of the egg-white, for it was found that a sample of egg-albumin was without this action. Perhaps the activator present is utilised in the metabolism of the fat and lipins of the egg-yolk during the development of the embryo. In order to test whether the action of "colloidal iron" is associated with its potential oxidising capacities, tests were carried out with dilute solutions of hydrogen peroxide 1/4000, ferric chloride 0·1 % and $N/400$ potassium permanganate. None of these had any action in activating pro-lipase.

2. *Action of inorganic compounds on pro-lipase.*

It had been found by Corran and Lewis [1928] that colloidal lead and lead salts, *e.g.* lead acetate, were capable of augmenting lipase action. It was not demonstrated, however, whether this was the result of some reaction with the blood-serum, with the oil emulsion or with one or both of the constituents of the lipase complex.

We have found that some lead salts have a definite activating effect on pro-lipase. In this they contrast with various other heavy metal salts *etc.*, which were tested in various concentrations, *e.g.* cuprous acetate, cupric chloride, thallium acetate, uranium acetate, selenious acid, cadmium chloride, thus confirming Corran and Lewis [1928], who found that copper and mercury salts had an inhibiting effect on lipase in absence of serum and in presence of serum or co-enzyme (Table II).

The experiments show that the sum of the separate activating effects of lead *plus* serum is somewhat greater than their combined augmenting action.

It is of interest that Cooper and Nicholas [1930] obtained somewhat different results using castor oil seed (lipase liberated by dilute acetic acid). In their experiments lead acetate or chloride in dilutions from 1/5000 to 1/5,000,000 had no effect on the reaction, mercuric chloride and copper acetate inhibited, whilst

Table II.

Pro-lipase cc.	Ox serum cc.	Salt 1 % solution cc.	Water cc.	Olive oil emulsion cc.	Titration cc. $N/10$ NaOH
—	—	1·0 lead acetate	1·0	4·0	0·7
0·5	—	—	1·5	4·0	1·2
0·5	0·5	—	1·0	4·0	26·8
0·5	0·5	1·0 lead acetate	0·5	4·0	33·4
0·5	—	1·0 „	0·5	4·0	13·4
0·5	—	1·0 lead chloride	0·5	4·0	12·2
0·5	—	1·0 cuprous chloride	0·5	4·0	0·5
0·5	—	0·5 thallium acetate	1·0	4·0	0·5
0·5	—	1·0 uranyl acetate	0·5	4·0	1·8
0·5	—	1·0 cupric chloride	0·5	4·0	1·4
0·5	—	1·0 $Co(NO_3)_2$	0·5	4·0	14·8
0·5	—	1·0 $MnCl_2$	0·5	4·0	3·0

lead glycine, lead triethyl chloride, thallium acetate or sulphate accelerated the lipolysis.

Thus it must be concluded either that the lipase is different from pancreatic lipase or that the reaction is affected by the substrate. In a succeeding portion of this paper it will be shown that such a change of substrate may materially alter the lipolytic action of the enzyme. On the other hand, evidence that the lipases in different organs have different characters has been given by many workers and is also afforded by such differences in physical constants as the following [Waldschmidt-Leitz, 1929].

	Pancreatic lipase	Ricin lipase	Gastric lipase
Opt. p_H	8·0	4·7	4–5
Temp. coeff. of reaction (20–30°)	1·26	1·23	—

3. *Action of salts of organic acids.*

The augmenting effect of sodium oleate was compared with that of a variety of organic salts, in order to ascertain to what degree this action (which had been found to be exerted through the substrate) was possessed by salts of various organic acids. Some of the unsaturated dibasic acids have been stated to play a definite rôle in the lysis of cancer cells [Freund and Kaminer, 1923] and might possibly do this by affecting lipolytic processes in these circumstances. It has also been stated that injections of oleic and linoleic acids and the sodium salts of these acids into mice caused an immunising action towards grafted tumours [Nakahara, 1924; 1925]. Since sodium oleate and stearate possess special properties of forming large micelles and colloid complexes, and thus differ materially from solutions of such salts as sodium acetate or sodium lactate, it would be expected that marked divergencies would be observed in their effects on the substrate and thus on the lipolytic action.

The following acids were tested, 1 % solutions of the sodium salts being utilised in all cases: lactic, citric, caproic, acetic, *n*-butyric, aminoacetic, maleic, tartaric, pyruvic, glycollic, malic, tiglic, ricinoleic, palmitic, stearic, sebacic, taurocholic, linoleic and glycerophosphoric.

In the first place none of these salts had any effect in activating pro-lipase, except taurocholate. This was a specimen of crude sodium taurocholate and caused a definite augmentation both with diluted aqueous extract and with flocculated pro-lipase (Table III *a*).

A number of these tests are illustrated in Table III *a*.

Table III *a*. *Effect of sodium salts of organic acids on inactive pro-lipase.*

(1 cc. of 1 % solution of salt for each test.)

Pro-lipase cc.	Organic salt	Water cc.	Olive oil emulsion cc.	Titration cc. *N*/10 NaOH
0·5 dil. extract	—	1·5	4·0	1·2
0·5 „	Na oleate	0·5	4·0	1·3
0·5 „	Na palmitate	0·5	4·0	0·8
0·5 „	Na citrate	0·5	4·0	2·0
0·5 „	Na taurocholate	0·5	4·0	7·8
1·0 pro-lipase	Na taurocholate	0·5	4·0	11·6
1·0 „	—	1·5	4·0	1·3

Many of these salts were capable, however, of augmenting the action of aqueous pancreatic lipase already lipolytic by reason of the presence of the natural co-enzyme. This is illustrated in Table III *b*.

Table III *b*.

Organic salts 1 % solution	Titration cc. *N*/10 NaOH
Control	38·0
Sodium oleate	47·3
„ acetate	50·4
„ citrate	53·6
„ lactate	51·1
„ ricinoleate	41·1
„ palmitate	42·2
„ stearate	38·6
„ linoleate	42·4
„ sebacate	48·8

The solutions were prepared from samples of pure B.D.H. acids and in all cases were adjusted so that they were "neutral" to phenolphthalein in presence of a mixture of alcohol and ether. No marked change was observed when, in a number of instances, potassium or calcium salts were substituted for the sodium salts.

Table III *c*. *Effect of sodium oleate on lipolysis of olive oil in presence of serum.*

Aqueous lipase cc.	1 % Na oleate cc.	Olive oil cc.	Ox serum cc.	Titration cc. *N*/10 NaOH
0·5	—	4·0	—	38·0
0·5	1·0	4·0	—	47·7
0·5	—	4·0	0·5	54·3
0·5	1·0	4·0	0·5	58·2

Since many of these organic acid salts caused an augmentation in lipolysis as great as that of sodium oleate it appears that their effects must be ascribed to the influence on the olive oil, *e.g.* by stabilisation of the emulsion.

Experiments carried out with a view to testing whether sodium oleate or other organic acid salts had, in addition, any direct effect on the activator of serum were inconclusive; the serum itself appears to help the emulsification of the oil very materially. This effect is indicated in Table III *c*.

4. *The action of pancreatic lipase and pro-lipase on neutral animal and vegetable oils.*

Olive oil forms an admirable substrate for testing the lipolytic activity of extracts, especially when purified and emulsified as described; it was thought to be worth investigating, however, whether various other natural fats and oils of similar composition behaved similarly towards the enzyme material. Samples of good quality oils, before and after treatment to remove traces of acid, were tested to compare their responses to the lipase. Table IV shows the results employing a potent aqueous extract, 0·5 cc. of enzyme, 4·0 cc. oil and 1·5 cc. water being used in all tests, incubation being for 48 hours.

Table IV. *Lipolysis of various oils by aqueous pancreatic lipase.*

	Lipolysis—cc. *N*/10 NaOH	
Oil	Crude	Purified
Coco-nut	71·8	66·8
Linseed	64·8	30·3
Castor	60·6	62·4
Palm	55·6	41·2
Hog lard	53·5	52·9
Cottonseed	50·6	44·4
Olive oil emulsion	46·8	—
Croton	45·0	28·2
Olive	40·8	48·0
Rape	41·0	41·2
Neatsfoot	38·6	40·4
Cod-liver	34·0	6·9
Chaulmoogra	25·2	58·0

It is evident that most of the above fats and oils are very sensitive to the action of lipase, many giving higher values of liberated acid than olive oil. The chief exceptions were cod-liver oil and crude chaulmoogra oil. The latter was somewhat acid and much greater lipolysis was obtained on using the purified material.

The low value with cod-liver oil which was invariably obtained is remarkable. It should be mentioned that this oil, even when treated with alkali, had a high content of cholesterol.

Experiments were made to test the effect of such dissolved cholesterol by making solutions of 1 % and 5 % pure cholesterol in warm olive oil. It was found that these solutions were not acted upon by pro-lipase. Also no lipolysis by pro-lipase was occasioned by the presence of 1 cc. of a cholesterol suspension (produced by dropping a 1 % alcoholic solution into water) when either olive oil or olive oil emulsion was used as substrate.

With glycerol lipase extracts Corran [1929] found that a coarse suspension of cholesterol acted as an augmentor. We also found that our colloidal suspension behaved as an augmentor to aqueous lipase extracts acting on olive oil emulsion. The low value of the lipolysis with the cod-liver oil is not attributable therefore to the presence of this impurity.

It is of interest that Ezoe [1929] found that the enzyme in spleen extracts digested oils in the following order: olive, sesame, rape, peanut, linseed, palm, whale, lard, castor, sardine, walnut, tripalmitin. This differs markedly from the order obtained with the pancreatic extract.

The action of pro-lipase, in presence and in absence of activator in the form of blood-serum was also tested on this series of fatty oils.

It was found that some of the unpurified oils were hydrolysed by the pro-lipase in absence of serum, suggesting that they contained activator themselves. It will be observed from Table V, that whereas the pro-lipase was inert towards hog lard, cod-liver oil, olive oil, croton oil and rape oil, a distinct reaction resulted with chaulmoogra, palm, coco-nut, neatsfoot, linseed and cottonseed oils.

Table V. *Lipolysis of oils by pro-lipase in presence and absence of activator.*

0·5 cc. pro-lipase emulsion. 4·0 cc. oil. 0·5 cc. ox serum.
Incubated for 48 hours with shaking.

Oil	Titration cc. *N*/10 NaOH	
	Serum present	Serum absent
Lard	44·5 (33·2)	2·2 (3·0)
Cod-liver	39·7 (17·8)	3·5 (2·4)
Croton	35·7 (21·7)	4·0 (2·6)
Olive	46·8 (42·8)	5·4 (5·0)
Rape	38·3 (42·5)	6·3 (5·2)
Neatsfoot	46·4 (38·4)	32·5 (3·9)
Chaulmoogra	31·0 (38·9)	27·3 (24·7)
Palm	45·2 (49·3)	27·2 (26·3)
Coco-nut	66·2 (62·5)	56·0 (23·4)
Linseed	50·0 (45·5)	45·0 (18·5)
Cottonseed	47·7 (39·7)	38·7 (10·1)
Castor	46·9 (36·6)	31·3 (29·3)
Human "depôt fat"	43·6	32·5

The figures in brackets were obtained after washing the oils with 1 % NaOH and then with hot water until the washings were neutral to phenolphthalein.

In some instances the washing apparently removed an appreciable amount of some activating substance, but in the cases of castor and chaulmoogra oils little change in the lipolysis ensued. Either, therefore, these oils retain the activator with very great tenacity or we have the remarkable instance of a form of lipase which is quite inert towards one group of neutral oils yet definitely active towards another series.

This possibility is of interest in connection with tumour formation since certain disturbances in metabolism might result in the formation of types of fats which would be more liable to hydrolysis and resynthesis than normal animal fats, thus causing further disorder in cell and tissue economy.

Ovarian dermoid cysts have been found to contain constituents such as cetyl or citosyl alcohol and cholesterol isomerides foreign to normal tissues. Also it appears that in some cases of malignant tumours the fats of the neoplasm are not available for general metabolism. Hirsch and Wells [1920] described a case in which a liposarcoma weighing 69 lbs. and containing 2 lbs. of fat was removed from a body in which normal fat depôts were completely exhausted.

5. *The activation of pro-lipase by physiological fluids and extracts.*

(*a*) *Heat-coagulated serum-protein.* By precipitating the proteins by heat-coagulation Corran [1929] found that the albumin and pseudoglobulin fractions of serum contained 80 % of the co-enzyme activity of blood-serum and that the dialysate was inactive. By first diluting blood-serum with 5 volumes of water, bringing to the isoelectric point with dilute acetic acid and then precipitating the proteins by heat we observed that the clear fluid obtained after removing the clotted proteins and concentrating had definite activating properties (Table VI, fluid 1). A portion of this fluid was dialysed against running water

Table VI. *Activation of pro-lipase by serum-proteins and physiological fluids.*

Specimen	Activation value cc. $N/10$ NaOH
Serum fluid 1	31·3
,, ,, 2	17·1
,, dialysate	12·9
,, fluid 3	16·3
Alcohol-precipitated protein	26·0
Normal urine	24·1
,, cerebro-spinal fluid	21·2
Ovarian fluid	26·3

for 36 hours after which it was found to have decreased in activating power by 50 % (Table VI, fluid 2). By dialysing a further portion against 500 cc. of distilled water for 24 hours and concentrating the dialysate to 20 cc. the passage of the activator through the membrane was also demonstrated (Table VI).

(*b*) *Alcohol-precipitated proteins.* The precipitate obtained by running 5 cc. of serum into 50 cc. of 50 % alcohol was washed with distilled water and made into emulsion with 20 cc. of water and tested for activator. The alcoholic fluid which was removed was concentrated to 10 cc. and also tested (Table VI, fluid 3). Both fractions gave a positive result.

(*c*) *Urine, cerebro-spinal fluid and ovarian fluid.* The activation values for specimens of urine, cerebro-spinal fluid and ovarian fluid are also given in Table VI. They were all potent activators; none possessed any intrinsic lipolytic activity. The urine and cerebro-spinal fluid were normal samples while the ovarian liquor was obtained from a cystic ovary removed at an operation and kindly supplied by Prof. Beckwith Whitehouse.

6. *Activation of normal and cancerous human blood-sera.*

Tables VII and VIII give the activation values obtained in a series of normal human sera and in sera from cancer patients respectively, using the quantities of reagents described previously. The normal sera gave high values (30–40 cc. $N/10$ NaOH) for the activation. It will be noted that the figures for

Table VII. *Activation values of normal sera.*

Blood sample	cc. $N/10$ NaOH
1	37·0
2	39·9
3	39·6
4	39·0
5	38·0
6	35·6
7	40·7
8	39·3
9	43·0
10	37·3
11	38·8
12	31·8
13	36·3
14	35·6
15	34·1
16	38·0
17	38·8
18	36·3
Mean	37·7

Table VIII. *Activation values of sera from cancer patients.*

Blood sample	cc. $N/10$ NaOH
1	38·8
2	31·8
3	36·3
4	30·6
5	31·6
6	37·3
7	38·0
8	35·9
9	39·4
10	41·3
11	40·6
12	31·8
13	34·8
14	34·9
15	35·8
16	36·8
17	37·2
18	39·8
19	35·1
20	35·3
21	36·6
22	35·1
23	33·5
24	33·0
Mean	35·5

the cancer sera are also consistently high and no very marked differences are to be observed between the individual figures or the average values. The majority of these cancer sera were obtained from pre-operative cases and from

a variety of types of malignant disease, diagnosis being made by members of the Honorary Staff of the Birmingham General Hospital.

The examination of a considerable number of specimens has now been carried out and the results confirm those indicated in the series quoted; the values obtained show that no specific alteration in the activation value of the blood-serum for pro-lipase occurs in cancerous conditions.

Certain observations made during the course of the investigation suggest, however, that it would be of value to examine the effect of lead and other metallic salts on the pro-lipase content of normal and tumour tissue.

Summary and conclusions.

For the purpose of investigating the properties of normal and cancerous serum in respect of their activating powers towards lipolytic pancreas extracts, a method has been elaborated for the separation of the inactive pro-lipase fraction of pancreas in sufficient quantities for the experimental examination of its interaction with chemical compounds, and with serum-co-enzyme and other physiological fluids.

A quantitative comparison of the lipolysis of various animal and vegetable fats by aqueous pancreatic extract and by this special form of flocculated pro-lipase has been carried out. Whereas pro-lipase is inert towards cod-liver oil, olive oil, hog lard and croton oil it readily decomposes palm oil, chaulmoogra and coco-nut oils even when these have been purified.

A co-enzyme capable of activating the pro-lipase is present in urine and in ovarian fluid.

Very small amounts of human and animal serum are capable of activating pro-lipase to the maximum extent. According to the results with a large number of sera obtained from varied types of malignant disease, and contrary to the opinion of some previous workers, the serum of cancer patients was just as efficient in activating the lipase as that from normal persons.

It has been observed that certain lead salts are able to activate the pro-lipase to an appreciable extent. This property is almost unique among the metallic compounds tested. The observations made in this investigation suggest that an examination of the pro-lipase content of tumours and the influence of lead and other metallic salts thereon would be of interest.

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CLXXX. THE EFFECT OF INTRAVENOUS PHOSPHATIDE ON THE SUGAR EXCRETION OF THE PHLORIDZINISED DOG.

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Parenteral injection of fats has never been a successful procedure. Failure of absorption is at least partially due to the physical properties of these substances. Fatty acids and their glycerol esters are notoriously insoluble in water, consequently, subcutaneous injection is followed by only very partial absorption and, usually, by the production of a foreign body tumour. Intravenous injection is seldom well borne because of emboli and further because it can readily be shown that many of the fat particles must be phagocytosed by the reticulo-endothelial system to remove them from the blood. They appear to act usually more as foreign bodies than as metabolites.

Conversion of fatty acid into the soluble sodium or organic base (mono, di, triethanolamine) salt suggests itself as a possible mode for the organism to deal with these insoluble substances. From the work of Page and Allen [1930], however, it appears improbable that the bulk of lipin could be transported or utilised in this manner. Actually the animal organism appears to make use of the solubility conferred by esterification of fatty acid with glycerol-phosphoric acid-base, the combination being known as phosphatide.

Evidence gathered by Page and Pasternak [1932] indicates that of all lipins studied, phosphatide was most easily introduced into the body. They found that after the intravenous injection of phosphatide (mainly kephalin) in amounts not exceeding 1·2 g./kg. into the rabbit, that the phosphatide could be recovered only in relatively small amounts from the blood or tissues. The liver was the organ most enriched by the injection. Subcutaneously or intraperitoneally injected phosphatide is rapidly absorbed if given in the proper emulsion form. Whether the material is actually burned remains an open question.

We were much interested in a recent report by Jost [1931], that phosphatide, especially kephalin from heart, seemed to be converted into sugar by the perfused dog liver. The sugar production of the liver of a dog starved and phloridzinised for four days in two experiments averaged 215 mg./1000 cc.

[1] Sharpey scholar.

blood after the injection of 50 cc. Ringer solution. In five experiments in which 5–8 g. of kephalin were perfused, the sugar rose after the phosphatide injection to an average of 448 mg. The perfusion volume of blood was 1600 cc.; therefore, subtracting the amount of sugar produced by the control, 215 mg., from 448 mg. there remains 233 mg. per litre or 372 mg. for 1600 cc. When as much as 8 g. of kephalin were employed for the perfusion it is not clear, however, why the glycerol or some contaminant could not account for this extra sugar. Jost may be perfectly correct in his interpretation but at the moment it is not convincing to us. The phloridzinised dog seems an excellent means of testing the hypothesis of the conversion of phosphatide into sugar, therefore we have undertaken these experiments.

Methods.

Phosphatide. The mixed phosphatide was prepared from human brain according to a method already described in detail [Rudy and Page, 1930; Page and Bülow, 1931]. Cerebrosides were eliminated but no effort was made to separate lecithin from kephalin. The latter substance predominates in such a mixture. 10 % water emulsions were easily made from the partially dried material. Iodine number and neutralisation values were not markedly affected by this procedure[1].

Phloridzinised dogs. The dogs were kept in metabolism cages and the urine was collected every 24 hours under toluene. For the first 48 hours the animals were starved and then allowed 100 g. of cooked liver daily. Estimations on the urine were not made until the fifth day which is given as the first day in the protocols. 1 g. of phloridzin (B.D.H.) was suspended in 7 cc. of warm sterile olive oil, well ground in a mortar and daily injected subcutaneously. Female dogs were used from which the labia had been removed. We are very grateful to Dr E. B. Verney for these animals. At the end of the 24-hour periods the residual urine in the bladder was removed by catheterisation.

Injections of phosphatide or glucose were made into a cannulated femoral vein. The introduction of the cannula was performed under the influence of the local anaesthetic percain. Injection of 100 cc. of fluid usually required 1·5 hours.

Chemical procedures. Nitrogen was determined by the Kjeldahl method on 5 cc. of urine. Glucose was estimated by the Benedict method on urine diluted 25 times with water. All reagents were standardised.

Results.

The results obtained with three of the seven animals treated are quoted and seem to us quite definite.

[1] We wish to thank Frl Dr M. Bülow and Frl L. Pasternak of the Chemische Abteilung, Kaiser Wilhelm Institut, Munich, Germany, for this material.

	Day	Urine volume cc.	Glucose g.	Nitrogen g.	D/N	Remarks
Dog 1	1	615	34·10	6·60	5·17	
	2	380	25·95	6·36	4·08	
	3	350	20·85	5·40	3·86	
	4	460	25·85	7·93	3·26	
	5	385	21·85	6·58	3·32	
	6	456	23·80	6·60	3·60	
	7	719	25·20	7·10	3·55	
	8	849	23·80	6·65	3·58	
	9	755	23·10	6·22	3·71	100 cc. 10 % glucose injected on 9th day
	10	850	30·25	7·08	4·27	100 cc. 10 % phosphatide injected on 10th day
	11	715	21·90	6·04	3·63	Dog killed on 11th day *Post mortem* revealed large abscess spreading over whole of back
Dog 2	1	340	38·80	8·25	4·70	
	2	492	49·50	10·59	4·68	
	3	543	53·80	11·74	4·58	
	4	327	36·30	6·79	5·35	
	5	386	36·80	11·20	3·29	
	6	253	25·30	6·97	3·63	
	7	262	26·80	7·42	3·61	
	8	293	26·60	7·28	3·66	100 cc. 10 % glucose injected intravenously on 8th day
	9	643	34·80	7·40	4·70	
	10	454	27·10	7·10	3·82	70 cc. of 10 % phosphatide injected intravenously on 10th day
	11	550	29·55	7·85	3·76	
	12	295	29·80	8·64	3·45	Dog killed on 12th day *Post mortem* revealed abscess on back
Dog 3	1	459	33·20	6·74	4·93	
	2	489	40·60	13·70	2·96	
	3	545	31·00	11·35	2·73	
	4	453	28·00	7·50	3·73	
	5	386	27·50	7·00	3·93	
	6	480	28·00	7·46	3·75	
	7	498	28·00	8·00	3·50	
	8	355	29·80	8·20	3·64	
	9	515	29·40	8·40	3·50	100 cc. 10 % glucose injected intravenously on 9th day
	10	565	38·20	9·30	4·10	
	11	958	31·90	8·86	3·60	
	12	582	27·10	8·03	3·37	
	13	535	27·90	7·83	3·56	100 cc. 10 % phosphatide injected intravenously on 13th day
	14	364	28·60	7·90	3·62	
	15	485	28·70	7·80	3·68	Dog killed on 15th day

Discussion.

It will be noted that the D/N ratios are considerably higher than those which have been reported in the past few years. This question has formed the subject of a separate communication and will not be discussed further than to state that these high ratios are associated quite specifically with the feeding of liver. Dr Stanley Benedict has been good enough to verify these results.

Glucose injected as a control for the recovery of added sugar was excreted adequately, and a corresponding rise in D/N ratio was observed. The ratios were also quite constant so that we believed that should extra sugar be formed

from a phosphatide injection in the phloridzinised animal we should be able to detect it. It seems clear from the above results that injection of phosphatide does not increase the extra sugar. There is only the slightest suggestion that any rise in D/N ratio occurs and this, if it is a real effect, might well be due to the conversion of glycerol into sugar.

SUMMARY.

Injection of aqueous brain phosphatide emulsion into phloridzinised dogs does not appear to alter the D/N ratio of the urine appreciably. As far as the phloridzinised animals are concerned, under the conditions of our experiment, there does not seem to be any conversion of fatty acid into sugar.

We are indebted to Prof. C. Lovatt Evans for helpful advice and criticism, and one of us also (I. H. P.) for hospitality in this Department.

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CLXXXI. CHEESE-RIPENING STUDIES[1].
II. THE NITROGEN REQUIREMENTS OF THE LACTIC ACID BACTERIA.

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In a previous paper [1932] we have reported upon the nitrogen distribution in forty-three nitrogen sources and have shown the influence of each of thirty-seven of these defined sources on the fermentation of glucose, mannose and lactose by five strains of lactic acid streptococci. The work confirms the findings of Orla-Jensen [1919] as to the importance of assuring a suitable nitrogen source in fermentation studies on the lactic acid bacteria, draws attention to the significant differences in the nitrogen distribution in certain of the sources we have investigated, and indicates clearly that in fermentation studies on the lactic acid streptococci the "kind" and the "amount" of nitrogen are both critical [see Eagles and Sadler, 1932].

In the light of these results we have studied the influence of four of the more significant of these nitrogen sources on the sugar-fermenting ability of thirty-three strains of lactic acid streptococci.

Experimental.

We have employed nitrogen sources 1, 28, 30 and 34 (see Table I), the broth in each case being prepared to contain 1 %, or approximately 1 %, total nitrogen. The distribution of the nitrogen in the four sources is shown in Table I, quoted from our previous paper.

Table I.

Nitrogen source	Protein-N	Proteose-N	Peptone-N	Sub-peptone-N
1 Peptic caseinogen digest	4·76	57·69	20·36	17·19
28 Proteose peptone (Difco)	0·0	29·68	47·84	22·48
30 Hydrolysed caseinogen (Difco)	4·82	35·84	27·26	32·08
34 "Bacto yeast extract"	0·0	0·0	24·20	75·80

The thirty-three strains of streptococci had been isolated from a Kingston cheese two weeks old. At the time of isolating, all colonies from one plate of a specific dilution had been picked off. Of the 54 colonies then recovered in pure culture, 33 only were available after a lapse of two years. It is unfortunate that

[1] These studies are provided for by a Research Fund established jointly by the Empire Marketing Board and the University of British Columbia.

Table II. *Titratable acidity in g. lactic acid per litre.*

Culture No. EMB$_1$	Xylose				Mannitol				Fructose				Glucose				Mannose				Galactose				Sucrose				Maltose				Lactose				Raffinose				Salicin			
	Nitrogen sources (see Table I)																																											
	1	28	30	34	1	28	30	34	1	28	30	34	1	28	30	34	1	28	30	34	1	28	30	34	1	28	30	34	1	28	30	34	1	28	30	34	1	28	30	34	1	28	30	34
172	0·7	0·5	2·3	1·6	0·7	4·1	3·2	0·9	0·2	6·8	0·7	1·1	0·2	6·1	7·0	2·5	0·2	5·6	5·9	1·4	5·9	0·7	3·4	1·8	0·0	0·0	0·2	2·1	2·7	0·2	0·7	1·6	6·8	5·9	0·5	0·2	0·5	0·0	0·5	0·0	0·0	0·2	0·0	0·9
173	0·7	0·5	1·6	1·1	2·7	4·3	4·5	0·2	7·4	7·2	8·1	3·4	8·6	7·0	7·4	2·0	7·4	6·5	6·8	2·5	7·2	5·2	4·5	1·8	1·1	0·5	0·2	2·5	1·1	0·5	0·7	1·4	7·9	6·5	7·2	2·0	1·1	0·2	0·9	0·0	6·5	6·3	7·7	0·9
174	0·9	0·0	1·4	0·7	0·7	0·2	0·2	0·2	7·2	6·1	6·5	3·4	7·2	5·6	5·4	1·8	6·3	5·2	5·4	1·8	5·9	4·5	3·4	1·1	0·9	0·0	0·2	1·1	0·7	0·0	0·5	1·4	6·8	5·4	6·1	1·4	0·5	0·2	0·5	0·0	5·0	3·6	0·0	0·7
175	1·4	0·7	2·9	0·0	0·5	0·7	0·5	1·4	7·9	6·3	7·2	1·6	9·2	7·0	7·4	0·9	8·6	7·0	7·0	0·7	7·2	5·2	4·3	0·0	0·9	0·0	0·0	0·0	1·1	0·2	1·1	0·2	7·9	6·8	7·0	2·0	0·9	0·0	0·5	0·2	7·7	6·3	0·5	0·5
176	1·1	0·2	1·4	1·4	1·1	0·5	0·5	1·1	8·1	6·3	8·3	4·1	8·6	6·8	6·8	2·5	7·9	6·3	6·1	1·1	6·5	4·7	3·2	1·8	0·9	0·0	0·0	0·5	1·1	2·0	0·5	1·8	7·2	6·1	6·5	3·2	0·9	0·0	0·9	0·5	7·0	6·3	5·0	1·6
178	1·4	0·9	2·5	0·5	0·2	0·5	0·7	0·5	8·1	6·8	8·1	0·2	9·5	7·9	7·9	2·7	8·6	7·0	7·4	0·7	5·4	0·9	0·9	1·4	0·7	0·7	0·2	0·2	0·7	0·7	—	1·1	1·1	0·5	1·1	0·7	0·7	0·2	0·7	0·7	0·2	0·5	0·5	1·8
179	1·4	0·0	0·9	1·6	0·5	0·5	0·9	0·2	7·9	6·5	8·3	3·4	8·1	5·9	5·9	2·5	7·2	6·1	6·5	1·1	0·9	4·7	5·0	2·0	0·9	0·0	0·7	0·7	0·9	0·0	—	1·8	7·4	5·6	6·8	3·4	0·7	0·0	0·9	0·5	5·0	4·3	0·7	1·1
180	0·9	0·5	1·6	1·4	0·5	0·7	0·7	0·0	8·6	6·5	7·9	1·1	9·5	7·4	7·9	2·0	8·3	6·8	7·4	0·7	7·2	5·0	4·3	1·8	0·9	0·9	0·5	0·0	0·9	0·7	—	1·6	7·7	6·8	7·4	2·0	0·9	0·2	0·5	0·0	0·9	0·5	0·2	0·5
181	0·0	0·0	1·1	0·9	0·0	0·0	0·2	0·2	0·5	0·5	0·5	1·8	6·5	5·2	5·9	2·0	0·5	0·5	0·7	0·5	2·0	5·2	4·1	1·6	0·5	0·2	0·0	0·5	0·7	0·5	0·2	1·6	2·5	5·9	5·9	0·2	0·5	0·0	0·2	0·2	0·0	0·0	0·2	0·5
182	0·2	0·5	0·5	0·0	0·5	0·5	0·9	0·0	0·5	0·7	0·9	0·5	0·2	0·2	0·0	0·9	0·2	0·5	0·5	0·5	0·9	0·7	0·0	0·7	0·5	0·2	0·5	0·2	0·5	0·2	0·5	0·0	0·5	0·5	0·5	0·0	0·9	0·2	0·7	0·0	0·5	0·7	0·7	1·4
183	0·7	0·2	0·9	1·1	0·0	0·2	0·0	0·2	7·7	6·8	8·1	3·2	9·0	7·2	7·0	2·0	8·1	7·0	7·2	1·1	6·8	5·4	4·5	0·2	0·9	0·0	0·0	0·9	1·1	1·6	0·2	1·6	8·1	6·5	6·1	3·6	0·5	0·2	0·2	1·1	6·8	5·0	0·2	1·6
184	1·8	0·0	0·5	0·9	2·5	4·3	1·1	1·8	8·1	6·8	7·4	1·8	8·6	6·8	6·8	0·5	8·6	7·2	6·5	0·9	7·7	5·2	4·1	0·7	1·1	0·0	0·7	0·0	0·0	0·5	1·4	1·6	8·1	6·5	6·5	3·2	0·9	0·0	0·9	0·7	6·8	5·0	4·1	1·6
185	1·1	0·5	2·0	1·1	0·5	0·5	0·7	1·1	7·7	6·8	7·2	3·4	8·1	6·3	6·1	3·4	7·2	6·1	6·5	3·6	6·5	4·7	4·1	0·9	0·7	0·2	0·2	0·9	0·9	0·2	0·5	2·5	7·2	5·9	6·1	3·4	0·7	0·0	0·2	0·9	6·1	3·2	5·2	1·8
188	0·9	0·2	1·6	1·8	0·2	0·0	0·2	1·4	0·2	0·5	0·7	1·8	7·2	6·3	7·0	2·5	0·5	0·2	0·9	1·6	2·0	2·3	0·5	1·6	0·5	0·0	0·2	1·4	0·5	0·5	0·5	2·0	1·8	6·1	5·2	0·5	0·2	0·2	0·2	1·1	0·2	0·2	0·0	1·6
189	0·9	0·0	1·1	1·6	0·5	0·9	0·7	1·6	8·6	7·2	7·4	1·6	8·1	6·3	5·9	2·5	7·4	6·1	6·1	1·8	6·5	3·6	1·1	1·1	0·7	0·7	0·5	1·1	4·5	1·1	4·5	2·9	7·7	6·5	7·0	3·8	0·5	0·5	0·0	0·9	2·3	1·1	0·5	1·1
190	0·0	0·0	1·6	1·4	0·0	0·9	2·3	0·0	7·7	6·1	5·4	2·3	7·7	5·4	6·5	1·8	7·2	3·4	5·0	0·2	4·1	0·0	0·5	0·2	4·1	4·3	7·2	3·6	4·3	5·6	5·6	2·3	0·0	0·0	0·0	0·0	0·2	0·0	0·5	0·0	0·0	1·8	0·0	0·0
191	0·9	0·2	1·6	0·7	0·7	0·5	0·7	1·1	8·1	6·1	7·7	3·8	8·8	6·8	6·8	1·6	8·6	6·5	6·3	1·6	7·4	5·2	3·6	1·6	0·9	0·0	0·0	1·6	1·1	0·0	—	2·3	7·7	6·1	7·0	2·7	0·9	0·0	0·5	2·0	7·7	4·5	0·2	1·4
192	0·7	0·0	1·4	0·0	0·5	0·5	0·7	0·9	8·3	7·0	7·2	1·6	9·5	7·4	7·0	2·5	8·6	7·0	6·3	0·9	6·3	1·4	0·5	1·4	1·1	0·7	0·7	0·9	0·7	0·9	0·5	1·1	1·1	1·6	1·6	0·9	0·7	0·7	0·7	1·1	0·5	0·9	0·7	0·9
193	1·4	0·7	1·6	2·9	0·7	0·7	0·7	1·1	8·3	6·8	8·1	2·9	9·5	7·4	8·1	2·3	8·8	7·4	7·9	0·0	8·3	5·6	5·9	0·2	1·1	0·0	0·5	6·3	1·4	0·0	1·8	0·5	8·6	7·0	7·2	3·4	0·7	0·0	0·5	1·1	7·0	5·4	4·1	1·8
195	0·7	0·0	1·1	1·6	3·8	3·8	3·6	0·7	8·1	7·4	8·3	2·7	8·3	7·0	6·5	0·5	7·9	6·3	6·1	1·4	6·8	4·5	3·6	1·4	1·1	0·2	0·7	0·0	1·1	0·0	2·0	1·6	7·7	6·5	6·3	2·3	1·1	0·0	0·7	0·0	6·5	—	5·0	0·7
196	1·1	0·0	1·6	0·7	0·5	0·0	0·2	0·2	7·9	6·3	7·4	1·1	9·0	7·4	7·4	2·3	8·1	5·0	—	1·1	6·8	5·4	0·9	1·1	2·9	0·7	0·5	0·0	0·7	0·7	0·2	1·4	0·7	0·7	0·9	0·2	0·5	0·0	0·2	0·9	0·2	0·5	0·2	0·9
197	1·4	0·5	2·3	1·6	0·7	0·5	0·7	1·4	10·1	8·1	9·2	1·1	9·9	7·7	7·9	2·3	8·8	7·0	7·9	1·4	8·6	5·0	6·1	1·4	1·1	0·2	0·5	0·9	1·1	0·7	0·7	2·3	9·0	7·0	8·1	4·1	0·9	0·2	0·5	1·4	0·9	0·7	0·5	2·0
200	1·4	0·2	1·4	0·5	0·5	0·2	0·5	0·7	8·6	6·3	7·2	1·0	9·2	7·0	7·4	2·0	8·1	6·5	7·2	1·1	7·4	4·3	3·6	0·9	0·9	0·2	0·7	1·1	0·9	0·5	0·5	1·6	8·3	1·8	6·3	4·1	0·9	0·0	0·5	1·6	3·2	0·7	0·2	1·6
201	1·1	0·0	0·9	1·6	0·7	0·5	0·7	0·7	9·0	7·7	8·6	2·7	9·2	7·4	5·9	1·8	8·1	6·3	7·2	0·7	7·0	4·7	5·9	0·0	0·9	0·7	0·5	0·5	1·6	0·7	0·9	2·5	8·6	6·8	7·7	2·7	0·7	0·2	0·5	1·1	0·5	0·7	0·5	0·7
203	0·9	0·2	1·1	2·3	0·2	0·2	0·2	1·6	0·5	0·2	0·7	2·0	6·8	5·6	6·3	2·3	0·5	0·5	0·2	1·8	4·7	4·7	4·3	1·6	0·7	0·5	0·2	1·6	0·5	0·5	0·2	2·3	6·1	5·2	5·4	0·7	0·2	0·2	0·2	0·5	0·2	0·2	0·2	1·1
204	1·4	0·7	2·3	0·7	0·7	0·2	0·7	0·7	8·8	6·5	7·7	1·6	9·2	7·0	7·9	1·1	8·6	6·8	7·0	0·7	7·7	5·4	5·4	0·9	0·9	0·5	0·7	0·5	0·9	0·7	—	1·1	8·3	6·8	7·7	3·4	0·9	0·2	0·9	0·2	1·4	0·5	0·2	1·1
205	0·9	0·2	2·3	0·9	0·2	0·2	0·2	0·0	8·3	8·1	7·4	0·5	9·0	8·1	7·7	1·4	8·1	7·9	7·0	0·0	7·4	6·3	5·0	0·5	0·7	0·2	0·0	0·9	0·9	0·7	0·5	1·4	7·9	7·9	7·0	0·7	0·7	0·2	0·2	0·7	3·2	4·3	0·2	0·7
206	1·6	0·7	4·1	0·9	3·2	4·1	1·6	0·0	8·1	7·0	7·2	4·3	7·9	6·5	6·3	2·3	7·2	5·9	6·1	0·9	6·8	4·5	4·1	0·9	1·1	0·5	0·2	0·5	1·1	0·2	1·1	1·8	7·2	5·6	6·3	2·5	0·0	0·2	0·5	1·1	5·0	3·2	0·7	1·6
208	0·9	0·0	0·9	0·2	0·2	0·2	0·0	0·2	7·7	6·1	7·0	0·7	8·6	7·0	7·0	0·2	8·3	6·3	6·1	0·0	7·0	4·7	3·4	1·4	0·9	0·2	0·2	0·2	0·7	0·5	0·5	1·1	7·9	6·8	4·7	0·2	0·5	0·0	0·2	0·0	0·2	0·5	0·2	0·5
209	1·4	0·9	2·3	1·8	0·5	0·5	0·7	2·0	8·6	7·7	8·3	2·7	8·3	6·5	7·0	2·3	7·7	6·5	7·0	1·6	6·3	3·2	0·7	0·0	0·9	0·5	0·5	1·6	4·3	1·1	2·3	2·7	7·9	6·3	7·7	3·8	0·7	0·0	0·5	0·2	3·6	4·5	0·2	1·4
210	1·4	1·1	1·8	1·4	0·5	0·2	0·2	1·1	8·3	7·7	8·1	0·0	8·6	5·6	7·0	1·6	7·9	6·1	6·8	0·7	6·3	3·6	1·6	1·1	0·7	0·7	0·2	0·7	5·0	1·6	1·6	2·3	7·9	6·1	7·0	3·2	0·2	0·2	0·2	0·7	3·4	4·3	0·0	1·4
216	1·1	0·0	1·1	0·7	0·2	0·2	0·5	0·7	0·7	0·7	0·9	0·7	7·2	6·1	6·1	1·4	0·5	0·7	1·8	0·0	2·3	5·9	5·2	0·0	0·9	0·2	0·2	0·5	0·7	0·5	0·7	0·2	2·3	6·3	5·9	1·4	0·2	0·2	0·5	0·5	0·2	0·5	0·0	1·8
220	1·4	0·2	2·3	2·3	0·7	0·5	0·2	1·6	9·9	7·9	9·2	2·0	9·9	7·4	7·7	2·0	8·6	6·8	7·9	0·7	8·1	3·8	1·6	2·5	1·1	0·5	0·0	0·9	1·1	0·7	—	2·3	9·0	6·8	7·9	3·4	0·7	0·0	0·2	0·9	0·7	0·5	0·2	2·0

among the strains lost were all those that responded appreciably to enrichment of media with yeast extract [Sadler, Eagles and Pendray, unpublished results]. The thirty-three cultures here reported upon are Gram-positive and appear in young milk culture as single cells, in pairs or as short or long chains. All fail to liquefy gelatin. The procedures followed in the fermentation study have been described in detail already [Orla-Jensen, 1919; Sadler, Eagles and Pendray, 1932].

The results of the determinations of the total titratable acidity—recorded as grams lactic acid per litre—produced by each of the thirty-three cultures from eleven carbohydrates when employing the nitrogen sources defined are given in Table II.

Discussion.

On examining and comparing the total titratable acidity figures obtained by using the four nitrogen sources respectively, yeast extract is seen to be an unsuitable source for the fermentation by these streptococci of fructose, glucose, mannose, galactose and lactose. In the fermentation of raffinose, however, when yeast extract is the nitrogen source, a few strains produce from 1 to 2 g. acid per litre; whereas all the organisms fail to produce as much as 1 g. acid per litre when the nitrogen is provided as caseinogen digest, proteose (Difco) peptone or hydrolysed caseinogen (Difco).

In the fermentation of xylose by certain strains there seems to be a significant value in yeast extract as a nitrogen source when compared with proteose peptone; but the superiority of hydrolysed caseinogen over any of the nitrogen sources is pronounced—the two most suitable sources being those containing a comparatively high and a very high percentage of the sub-peptone nitrogen fraction.

Mannitol is fermented to some small extent by many of the streptococci when the nitrogen is supplied as yeast extract; when proteose peptone is the source of nitrogen fewer strains are active but those that are active produce as much as 4 g. acid per litre. The influence of hydrolysed caseinogen is, in a measure, comparable with that of proteose peptone; but caseinogen digest appears to be unsuitable.

Except one strain, EMB_1 190, all the streptococci produce less than 1 g. acid per litre from sucrose when either hydrolysed caseinogen or proteose peptone is the nitrogen source. Caseinogen digest suits a few strains. When the nitrogen is supplied as yeast extract the total amount of acid produced is always low; but more of the cultures produce a titratable amount of acid if supplied with this source than is the case with any of the other nitrogen sources used.

In the fermentation of maltose, five strains produce over 2·7 g. acid per litre when the nitrogen is supplied as caseinogen digest and four strains produce over 2·0 g. when hydrolysed caseinogen is supplied. When the nitrogen source is yeast extract 18 strains produce from 1·0 to 2·0 g. acid per litre and

11 strains produce from 2·0 to 2·9 g. acid per litre. In the fermentation of maltose by these streptococci, yeast extract appears to have a significant value as a nitrogen source.

Few of the streptococci ferment salicin when the nitrogen is supplied as hydrolysed caseinogen. Many produce a small amount of acid when the nitrogen source is yeast extract. When the nitrogen is supplied as caseinogen digest a number of strains are inactive; but some twelve strains are vigorous—defining vigour and activity in terms of total titratable acidity produced. In lesser measure the influence of proteose peptone is comparable with that of caseinogen digest.

In considering the total titratable acidity produced by the streptococci from fructose, glucose, mannose, galactose and lactose, yeast extract as a source of nitrogen does not bear comparison with any of the other nitrogen sources employed. Almost all the strains produce a high total titratable acidity from these sugars when the nitrogen source is caseinogen digest, proteose peptone, or hydrolysed caseinogen. The activity of the organisms is most distinct, however, when the nitrogen is supplied as caseinogen digest, the superiority of this source being especially evident in the fermentation of galactose.

Considering the results as a whole, it is to be seen that when provided with yeast extract—a source containing 75 % sub-peptone-N, 25 % peptone-N and no proteose-N [Sadler, Eagles and Pendray, 1932]—many of the streptococci produce a small amount of acidity from most of the carbohydrates employed, the total titratable acidity never being high. In order to bring about active and vigorous fermentation of fructose, glucose, mannose, galactose and lactose these streptococci require a nitrogen source containing an appreciable amount of proteose-N, peptone-N and sub-peptone-N, and we have seen that these requirements are best met by using the peptic caseinogen digest.

Our results support Orla-Jensen [1919] in his insistence on the critical importance of the nitrogen source in fermentation studies on the lactic acid bacteria, emphasise the suitability of a peptic caseinogen digest—a suitability so clearly shown by Orla-Jensen and now shown by us in a still more specific sense—and confirm our previous findings.

The work would seem to suggest that the specific lactic acid streptococcus strains demand a certain moiety of a particular nitrogen fraction for the fermentation of a particular carbohydrate: that in some measure there is a protein-carbohydrate-streptococcus linking up. Furthermore, there is the suggestion that at least the variety of nitrogen fractions we have employed in this study might be used to advantage in studies on the sugar-fermenting abilities of the lactic acid streptococci.

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CLXXXII. p_H OF BLOOD OF PSYCHOTICS MEASURED BY THE GLASS ELECTRODE.

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THE constant p_H of the blood is normally dependent on two mechanisms, renal and respiratory. It has been shown by Golla *et al.* [1928], Golla [1929], Mann *et al.* [1928], Mann and Marsh [1928], Marsh [1929] and Robertson [1927] in a series of papers from this laboratory, that in the psychotic the renal mechanism is intact, but that there is a disturbed regulation of the respiratory mechanism for the control of acid-base equilibrium, as evidenced by delayed response to the stimulation of breathing CO_2-air mixtures. The object of the work described in this paper is to see whether such a tolerance of increased alveolar CO_2 pressure is reflected in a decreased blood-p_H either in the resting subject or when he is submitted to a stress.

The patients selected for experiment were certified cases of schizophrenia with a few other pathological cases for comparison. A series of normal controls was obtained and this was supplemented by discharged general paralytic patients all of whom were active and in reasonably good physical and mental condition.

For resting values of blood-p_H, the subject was always in the post-absorptive state and was either in bed, or had rested for at least 30 minutes.

The stress consisted, firstly, of ingestion of 20 g. of $NaHCO_3$ dissolved in half a pint of water, thereby causing a temporary increase in p_H, and secondly, of performing exercise by running or standing running, causing a temporary decrease in p_H.

EXPERIMENTAL.

Blood was obtained from the brachial artery, the median basilic vein and, in a few cases, from the jugular bulb. The samples were collected under paraffin in an all-glass syringe, on the plunger of which a trace of anticoagulant was placed. The blood was run immediately into tubes containing paraffin and a small amount of either heparin or Evans's 9 : 1 mixture of potassium oxalate and sodium fluoride, previously moistened with saline. After stirring the blood with a thin glass rod the tubes were placed in an ice-box.

The apparatus used for p_H measurements was the glass electrode outfit described by Kerridge [1926]. The glass electrode vessels were made from soft laboratory glass tubing of about 9 mm. diameter, the relatively wide opening enabling the lower chamber to be filled or emptied without the necessity of a second arm. After standing in cleaning mixture for a short time they were soaked in distilled water for several days and in some cases it was an advantage to leave them filled with phosphate buffer solution for a few hours before use.

The insulated stand holding the calomel and glass electrodes was contained in an ordinary incubator kept at 38° and connections were made by means of insulated cable wire running through the thermometer hole of the incubator. The inner casing of the incubator was earthed by means of wire connected to the water overflow pipe. This arrangement, whilst by no means so efficient as the large air-oven with fan as used by Kerridge, proved satisfactory for the technique which was adopted.

The calomel electrodes contained saturated KCl and considerable trouble was experienced at first from the crystallisation and creeping of KCl. The difficulty was overcome by allowing the ends of the tubes to dip into saturated KCl solution contained in wide tubes and bridging over from these to the glass electrode chambers by means of saturated KCl-agar tubes. This added to the speed with which it was possible to prepare the electrodes for successive observations and, since readings were made only to the nearest millivolt, did not entail any loss of accuracy.

In the time available, particularly if several determinations had to be carried out on the same day, it was impossible to wait until the blood, run at room temperature into the glass electrode chamber, had reached a temperature of 38°. In spite of the fact that the oven containing the electrode, although momentarily cooled when opened, quickly regained its original temperature, the blood took much longer. The further complication of the "first acid change," noted by Havard and Kerridge [1929], made it essential for readings to be made fairly quickly at temperatures approaching 38°.

It was found very convenient, especially in winter, to work at a blood temperature of 32° to 33°, equilibrium being reached rapidly. This required the application of the Kerridge correction of $-0{\cdot}015$ p_H per ° C., but had the advantage that it was possible to carry out duplicate determinations on a specimen of blood and the standard buffer solution, all in the same electrode.

The lower chamber of the glass electrode was filled with phosphate buffer solution of p_H about 6·0, and the standard buffer for comparison was always of a p_H to give a potentiometer reading approximating to that of the blood specimen. This was necessitated by the fact that the particular electrodes used seldom gave quite accurate readings when comparing two buffer solutions of widely varying p_H values. Thus, for p_H determinations of the blood of resting subjects, the standard buffer p_H was 7·40 at 33°. This was checked by means of the quinhydrone electrode.

The potentiometer reading for the buffer solution in a clean glass electrode often differed from that of the same solution in the same electrode contaminated with liquid paraffin. To minimise errors therefore the reading for the blood sample was taken first, followed by that of the standard buffer also delivered into the electrode under paraffin, and then a duplicate result was obtained for the blood sample. Hot distilled water was used for washing out the chamber between these operations. Under these circumstances the duplicate readings for the blood sample were taken as sufficiently accurate if they differed by not more than 1 mv., or 0·2 p_H. The results were calculated to 38° by the formula:

$$p_{H\ 38°} = p_{H\ \text{Buffer soln. } 33°} + \frac{(V_1 - V_2)}{60{\cdot}7} - 0{\cdot}075,$$

where V_1 is the potentiometer reading in mv. for the blood sample at 33°, and V_2 is that of the standard buffer solution at the same temperature.

On some of the specimens the alkali reserve of the plasma was determined by the indirect (back titration) method.

On the blood samples which were obtained after exercise, lactic acid determinations were carried out by the method of Friedemann, Cotonio and Shaffer [1927], modified by Friedemann and Kendall [1929], the apparatus used being that designed by Davenport and Cotonio [1927]. The percentage recovery of lactic acid was between 95 and 98. When sodium tungstate and sulphuric acid were used for deproteinising, it was found unnecessary to treat the filtrate with copper sulphate and lime, the error involved being negligible within the limits of accuracy of the experiment. This extra stage was, however, necessary when trichloroacetic acid was used as deproteinising agent.

Discussion.

The results obtained with resting subjects are shown in Table I. These show that on the average the p_H of the blood in schizophrenia is quite normal, although the variation appears to be greater than in the normal.

It should also be pointed out that some of the patients became very agitated when being prepared for puncture and this may have caused increased ventilation, with a corresponding small rise in p_H.

The figures for alkali reserve again show no departure, on the average, from the normal.

The next step was to determine the response to bicarbonate ingestion. The figures recorded in Table II show that here again the psychotic behaves in a normal manner, at least in the early stages after ingestion.

A very different condition is seen when the psychotic performs exercise. Many of the patients are, of course, badly out of training and the figures for lactic acid shown in Table III are in the majority of cases out of all proportion to the amount of work actually done. In some cases exercise had to take the form of being pulled along by a male attendant. In Case No. 48 a sample of

Table I. p_H *and (alkali reserve in vols. % CO_2) of blood of resting subjects.*

Case Nos.	Condition	Arterial	Venous
1–12	Normal, highest	7·39 (57·6)	7·35 (64·9)
	,, lowest	7·35 (53·5)	7·32 (60·5)
	,, mean	7·37 (55·7)	7·33 (62·5)
13–49	Schizophrenia, highest	7·44 (62·0)	7·39 (77·4)
	lowest	7·32 (50·0)	7·29 (57·2)
	mean	7·36 (55·6)	7·32 (63·5)
50–60	Miscellaneous:		
	Paranoia (H.G.D.), resting	7·37	7·32
	Induced tetany, before spasm	—	7·33
	,, ,, after ,,	—	7·35
	,, ,, before ,,	—	7·32
	,, ,, after ,,	—	7·37
	Myasthenia gravis, resting	—	7·33
	Epilepsy, immediately after fit	—	7·44
	Border line D.P., resting	7·31	—
	Imbecile, resting	7·35	—
	,, ,,	7·38	—
	Certified G.P.I.	—	7·32
	,, depressive	—	7·31
	Uraemia (severe)	7·25	—
	,, (recovering)	7·32	—

Table II. p_H *of venous blood before and after ingestion of $NaHCO_3$ (20 g.)*

	Case No.	Before	35 minutes after
Discharged G.P.I.	11	7·33	7·37 (10 g.)
,,	12	7·32	7·38 (20 g.)
Certified G.P.I.	58	7·32	7·37 ,,
Depression	61	7·31	7·36 ,,
Schizophrenia (active)	38	7·29	7·35 ,,
,, (stuporose)	39	7·30	7·33 ,,
,,	49	7·31	7·35 ,,

Table III. p_H *of venous blood before and after exercise.*

(Figures in parentheses refer to lactic acid content of whole blood in mg./100 cc.)

	Case No.		Resting	5 minutes after exercise
Normal	5	Standing running 1 minute (restrained)	7·33 (13)	7·27 (24)
,,	2	Standing running 1 minute (less restrained)	7·34 (10)	7·25 (40)
,,	4	Standing running 1 minute (all-out)	7·33 (13)	7·20 (79)
,,	4	,,	7·35 (12)	7·23 (53)
Schizophrenic	40	Ran with nurse	7·33 (13)	7·18 (84)
,,	42	Standing running 1 minute	7·33 (17)	7·20 (63)
,,	43	Ran with nurse	7·30 (13)	7·18 (57)
,,	45	,,	7·32 (11)	7·16 (97)
,,	47	,,	7·33 (12)	7·18 (88)
,,	64	Standing running 1 minute	7·31 (10)	7·25 (29)
,,	65	Ran with nurse	7·36 (10)	7·19 (59)
,,	48	,,	7·37 (23)	7·25 (50) (10 minutes after exercise)
Male nurse accompanying No. 48				7·30 (27) (8 minutes after exercise)

the attendant's blood taken after the exercise showed only half the lactic acid content of that of the patient. This patient and No. 45 were very agitated.

The length of time after exercise in which the blood-lactic acid concentration reaches a maximum depends on the duration and severity of the exercise. After standing running for 1 minute the maximum occurs between 3 and 7 minutes and for comparable results, therefore, the needle was inserted into an arm vein exactly 5 minutes after exercise. The results indicate that for a given amount of lactic acid in the blood after exercise the p_H is definitely below that of the normal. This is shown more clearly by the graphical representation in Fig. 1.

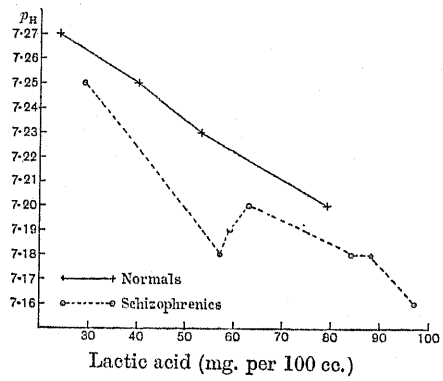


Fig. 1. p_H and lactic acid content of whole venous blood, 5 minutes after exercise.

In view of the normal figures for alkali reserve, this can only be accounted for by decreased ventilation on the part of the patient and supports the contention of Golla and Mann that in the psychotic there is a diminished excitability of the respiratory centre to carbon dioxide. It remains an open question, however, how far the results obtained could be interpreted purely on the ground of lack of training.

Summary.

1. The use of the glass electrode for semi-routine determinations of p_H is described.

2. The p_H of arterial and venous blood of normals and psychotic patients has been determined when the subject is in the resting condition and when he is submitted to the stress of alkali ingestion or of acid accumulation due to exercise.

3. The results, which are tabulated, show no significant difference of schizophrenics from the normal either in the resting condition or in the early stages after alkali ingestion.

4. It is shown that as a result of exercise the schizophrenic has a blood-p_H lower than that of the normal for a given lactic acid content, and it is argued that this is due to diminished excitability of the respiratory centre.

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CLXXXIII. VITAMIN A DEFICIENCY IN CASTRATED MALE RATS.

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In a previous paper [Sampson and Korenchevsky, 1932] it was shown on the basis of paired feeding experiments, that vitamin A deficiency produces changes in various organs and functions of male rats and in particular degeneration of the seminiferous tissue of the testis.

The present paper describes the study of the effect of vitamin A deficiency on castrated male rats, from which both the seminiferous and the interstitial tissues of the testis are absent, in an attempt to collect data for the elucidation of the possible interrelationship of vitamin A and the testicular hormones.

Technique.

Experiments were performed on 16 rats belonging to three litters. In litters Nos. 2 and 3 (Table I) the rats were divided into the following three groups: (i) castrated rats on an adequate, unlimited diet (CoU diet); (ii) castrated rats, on vitamin A-deficient diet (−A diet); and (iii) normal rats, on vitamin A-deficient diet. In litter No. 1 only the last two groups were present. All three litters were born on the same day. Some of the rats of each litter were castrated on the 22nd day after birth and the experimental feeding was started on the 31st day after birth.

The rats were killed on the dates given in Table I, so that in each litter, one rat of each group was sacrificed simultaneously. The only exception was rat

Table I. *Final weights of rats, their daily average food intakes per 200 g. of body-weight and average daily gains in body-weight per 10 g. of actual food intake (g.).*

	Castrated on CoU diet				Castrated on −A diet				Normal on −A diet				
No. of litter	No. of rat	Final body-wt.	Food in-take	Gain in wt.	No. of rat	Final body-wt.	Food in-take	Gain in wt.	No. of rat	Final body-wt.	Food in-take	Gain in wt.	Duration of exp. in days
1	—	—	—	—	1	142	26·6	0·9	4	175	26·1	1·2	48
	—	—	—	—	2	120	36·2	1·3	5	159	35·4	1·6	25
	—	—	—	—	3	140	23·1	0·6	6	143	29·0	1·1	70 & 40
2	7	201	33·1	2·0	9	147	31·2	1·5	11	157	31·5	1·3	34
	8	179	37·1	2·1	10	140	33·7	1·7	12	136	36·7	1·4	26
3	13	147	33·9	1·7	14	103	35·2	1·1	16	116	35·6	1·1	32
	—	—	—	—	15	102	34·3	1·1	—	—	—	—	32

No. 6 which was killed on the 40th day after being put on the experimental diet, while rat No. 3, the corresponding rat in the other group, was not killed until 30 days after. This was done because rat No. 3 was in good condition on the 40th day and it was decided to ascertain the length of time it would survive on the vitamin A-deficient diet.

The technique of the experiments was identical with that used in the previous investigation [Sampson and Korenchevsky, 1932] except that paired feeding was not used, as the influence of castration on the appetite of rats on a vitamin A-deficient diet was also investigated in the present experiments.

The rats were always killed on the day after the occurrence of the first significant decrease in appetite, so that they should not be investigated in the emaciated state due chiefly to a very decreased food intake.

Experimental results.

Changes in body-weight and appetite.

Although paired feeding was not used, the appetites of the groups of rats on −A diet which were killed at the same date, were nearly the same, *e.g.* rats 1 and 4, 2 and 5, 9 and 11, *etc.* (see Table I).

In some of the rats on CoU diet the appetite (per 200 g. of rat weight) was even less than that of those on −A diet, *e.g.* in litter No. 2, rat 7 ate less than rats 10 and 12; in litter No. 3, rat 13 ate less than any of the rats of this litter on −A diet (Nos. 14, 15 and 16). This is only the case when the food intake is calculated for the same unit of body-weight. In actual figures the appetite of the rats on CoU diet was larger than that of those on −A diet. For example, in the groups "castrated, on CoU diet," "castrated, on −A diet" and "normal, on −A diet," the average figures of the actual food intakes were in litter No. 2, 21·5, 17·9 and 19·6 respectively and in litter No. 3, 16·8, 14·8 and 16·3 respectively.

Since when these figures are calculated per 200 g. of body-weight this relationship is reversed for most rats, it is possible to draw conclusions, even from these experiments, about the anabolic properties of vitamin A, which have already been demonstrated and discussed in our previous paper [Sampson and Korenchevsky, 1932].

Thus for one unit of food intake the daily increase of body-weight of rat 8 was 2·1 g. whilst for the rats Nos. 10 and 12 on the −A diet it was 1·7 g. and 1·4 g. respectively.

In litter No. 3 the gain on the complete diet was 1·7 g. per unit of intake, while on the deficient diet it was 1·1 g.

These results, therefore, in agreement with those obtained previously, indicate an "anabolic principle" of vitamin A because of which one unit of adequate diet causes a greater increase in body-weight than the same unit of a diet deficient in vitamin A. No difference in this respect was noticed between the normal and the castrated rats. Thus, if rats 3 and 6 are excluded as having been

killed at different ages and rat 15 as having no normal rat on -A diet corresponding to it, the gain in body-weight per 10 g. of actual food intake for all the remaining rats was on the average 1·30 g. for the group of castrated rats on −A diet and 1·32 g. for the group of normal rats on −A diet, *i.e.* practically the same.

With the exception of two rats, killed 57 and 79 days after castration, all were killed 34 and 43 days after castration. It has already been shown [Korenchevsky, 1925, 1930] that some of the changes in castrated animals are not fully developed until some months after the operation. The absence of difference in the response of normal and castrated animals to vitamin A deficiency might disappear at a later period. The results obtained, therefore, can only be regarded as valid for the period about two months after castration.

Changes in organs.

In Table II are given the weights of those organs only in which constant and obvious changes were observed. In order to economise space the weights

Table II. *Average weight of organs of rats in each group of each litter (mg.).*

		Castrated on CoU diet Weight		Castrated on −A diet Weight		Normal on −A diet Weight	
Organs	No. of litter	Actual	Per 200 g. body-wt.	Actual	Per 200 g. body-wt.	Actual	Per 200 g. body-wt.
Penis	1	—	—	79	121	150	188
	2	53	56	51	71	137	188
	3	45	61	40	77	132	228
Av. of litters 2 and 3		49	59	46	74	135	208
Prostate and Sem. ves.	1	—	—	57	87	620	787
	2	65	70	67	93	355	475
	3	46	63	41	79	511	881
Av. of litters 2 and 3		56	67	54	86	433	678
Adrenals	1	—	—	42·8	65·3	36·8	46·6
	2	51·4	54·5	35·2	49·2	32·9	45·4
	3	49·4	67·2	25·8	50·2	26·9	46·4
Av. of litters 2 and 3		50·4	60·9	30·5	49·7	29·9	45·9
Pituitary	1	—	—	8·5	13·0	7·3	9·2
	2	10·3	10·9	8·5	11·8	7·3	9·9
	3	9·3	12·6	7·1	13·9	5·7	9·8
Av. of litters 2 and 3		9·8	11·8	7·8	12·9	6·5	9·9
Thymus	1	—	—	214	341	219	274
	2	518	544	454	632	242	332
	3	434	590	245	478	194	334
Av. of litters 2 and 3		476	567	350	555	218	333

of the organs of each rat are not included and only the average figures for each group in each litter given, for, as stated above, these changes were present in all the rats of the group.

Sexual organs. The penis, and prostate and seminal vesicles were much smaller in castrated than in normal animals. In the castrated animals while the average actual weights were nearly the same in both groups, when calculated ger 200 g. of body-weight, those of the rats on −A diet were heavier than those of the rats on complete diet. On a vitamin A-deficient diet, therefore, the atrophied sexual organs of castrated rats undergo the same type of changes in weight as those observed in the sexual organs of normal rats [Sampson and Korenchevsky, 1932].

Adrenals and hypophysis. These glands, as shown by the weights calculated per 200 g. of body-weight, when compared with those of normal animals, were hypertrophied in castrated rats. This change had previously been found in castrated rats on normal diet [Korenchevsky, 1930] and is also present in castrated rats on −A diet.

No constant or considerable difference in these glands was noticed between the two groups of castrated rats, except that in rats on −A diet hypertrophy of adrenals was less pronounced than that in rats on CoU diet.

Thymus. This gland, both in actual weight and calculated per 200 g. of body-weight, was larger in castrated animals, but no constant or considerable difference was noticed between the glands of the two groups of castrated rats.

Summary.

1. 16 male rats belonging to three litters were used in order to compare the influence of vitamin A deficiency on normal and castrated rats.

2. The conclusion, obtained previously on unoperated rats, was corroborated, namely that one unit of vitamin A-deficient diet produces a smaller increase in body-weight than the same unit of adequate diet.

3. Thus this fact, showing an anabolic principle of vitamin A, was also observed in castrated rats.

4. When calculated per unit of body-weight the atrophied sexual organs of castrated rats on −A diet were heavier than those of castrated rats on adequate diet. This change was previously observed in unoperated rats.

5. The adrenals and hypophysis of rats on −A diet undergo, after castration, a hypertrophy similar to that observed in castrated rats on normal diet.

6. Irrespective of the diet, the thymus was larger in castrated than in normal animals, but no essential difference was noted between the glands of castrated rats on adequate diet when compared with those on A-deficient diet.

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CLXXXIV. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

VII. THE ANAEROBIC CONVERSION OF FRUCTOSE INTO LACTIC ACID BY TUMOUR AND ADULT NORMAL TISSUES.

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It was shown in Part VI of this series [Dickens and Greville, 1932, 1] that whilst the various embryonic tissues all readily convert glucose into lactic acid, they differ in their behaviour towards fructose, their rate of attack on this sugar varying from zero to a value equal to that on glucose. The ability to convert fructose into lactic acid is thus not by any means an invariable property of rapidly growing normal tissues; and the varying behaviour of the different tissues towards fructose renders this sugar a valuable agent for the study of their carbohydrate metabolism. In the present communication we describe the anaerobic attack on fructose of the adult normal tissues on the one hand, and of the rapidly growing malignant tissues on the other.

Experiments were made on slices of surviving tissue by the method of Warburg; full details of technique have been given in Part VI. The terms *glycolysis*, *glucolysis*, *fructolysis*, *autoglycolysis* and *relative fructolysis*, which were defined and discussed in that paper, are used in the present description.

Normal tissues.

All measurements were made on the tissues of fed albino rats. The tissues are arranged according to the extent of their attack on glucose.

Liver.

Isolated adult liver tissue is remarkable in that it does not form lactic acid from glucose, the presence of which, even in high concentrations, has no appreciable effect on the anaerobic glycolysis; moreover, glucose does not affect the extra-fermentation caused by preliminary aerobiosis [Rosenthal, 1929; 1931]. On the other hand, it is well known that the liver may readily attack fructose [Oppenheimer, 1912; Rosenthal, 1930, 1, 2; 1931]. Rosenthal has made a thorough study of the anaerobic conversion of fructose into lactic acid by rat-liver; this only occurs in fed animals, in which it varies greatly

from animal to animal under identical external conditions, but is greatest on the whole in the summer months. On increasing the fructose concentration the fructolysis rises rapidly, reaching a maximum at about 1·6 %; and the rapid fall with time of fructolysis, which occurs during the first hour with liver in 0·2 % fructose, is prevented when the concentration is raised above 0·4 %. These latter observations we have confirmed. Thus for the demonstration of fructolysis in liver, fed animals in the summer months must be used, the fructose concentration may have to be raised, and the livers of several animals should be tested. These conditions have been borne in mind when studying the tissues described below, but in these we have found no evidence of the seasonal variation seen in liver.

Intermediate tissues.

The tissues of this group convert glucose into lactic acid, but not as readily as the nervous tissues.

Kidney usually attacks fructose slightly, as will be seen from Table I, in which the relative fructolysis for the second hour is given in the last column. Increase of fructose concentration has little, if any, effect. With submaxillary gland and spleen the anaerobic attack on fructose is negligible, but testis attacks the sugar slightly (Table I).

Table I. *Adult normal tissues* $Q_M^{N_2}$.

Date	Glucolysis 1st hr.	Glucolysis 2nd hr.	Fructolysis 1st hr.	Fructolysis 2nd hr.	Autoglycolysis 1st hr.	Autoglycolysis 2nd hr.	Relative fructolysis (2nd hr.) %
Kidney:							
27. i. 32	8·1	—	2·7	—	1·7	—	(16)
7. vii. 32	5·5	3·4	4·9	1·3	4·0	1·2	5
11. vii. 32	3·8	3·3	(0·2 %) 3·25	1·55	1·75	0·9	27
			(0·7 %) 3·4	1·6	—	—	29
			(1·5 %) 3·85	1·8	—	—	38
12. vii. 32	5·7	3·4	3·75	1·75	2·3	1·2	25
13. vii. 32	4·8	4·6	3·4	2·0	2·6	1·4	19
Submaxillary:							
7. vii. 32	8·3	6·9	2·6	0·6	3·7	0·5	0
12. vii. 32	11·7	9·9	2·3	1·1	2·7	1·0	0
Spleen:							
23. i. 32	—	—	1·6	—	0·9	—	—
7. vii. 32	7·4	6·7	3·3	1·8	2·7	1·5	7
12. vii. 32	12·0	11·4	2·2	1·8	2·3	1·5	3
Testis:							
23. i. 32	—	—	2·8	—	0·6	—	—
29. ii. 32	13·0	11·0	4·5	4·0	2·0	1·0	30
7. vii. 32	7·5	7·5	3·0	2·8	2·2	1·8	18
12. vii. 32	9·3	8·7	2·9	3·1	2·1	2·3	13
Brain:							
14. i. 32	13·8	9·2	1·1	0·7	1·0	0·1	7
			1·1	0·7	0·8	0·1	7
13. vii. 32	12·5	9·6	2·4	0·6	—	—	—
Retina:							
29. iii. 32	64·0	—	3·0	—	3·5	—	0
30. iii. 32	—	—	4·0	—	—	—	—

Nervous tissues.

These tissues show a high glucolysis, but they lack the power to convert fructose into lactic acid. This has already been shown for nerve [Gerard and Meyerhof, 1927] and brain [Loebel, 1925], with whose observations the figures for both winter and summer brain in Table I agree well. Retina does not show the slightest anaerobic attack on fructose, in spite of its great attack on glucose (Table I).

TUMOUR TISSUES.

Tumours of the rat, mouse, rabbit and fowl have been used in our experiments.

Rat tumours. Warburg *et al.* [1924] report on an anaerobic fructolysis of $Q_M^{N_2}$ 3·3 with the Flexner-Jobling carcinoma, the glucolysis being 23·9. Rosenthal [1930, 2] has made a number of observations with the Jensen rat sarcoma. He finds an anaerobic fructolysis equal to about one-sixth of the glucolysis, but some 45 minutes after the vessels are put in the thermostat the fructolysis rises, reaching a steady value about 30 minutes later. The rise amounts on an average to 60 %, varying between 0 and 135 %. We have confirmed the existence of this phenomenon in the Jensen sarcoma (see Fig. 1 (*a*)): as an

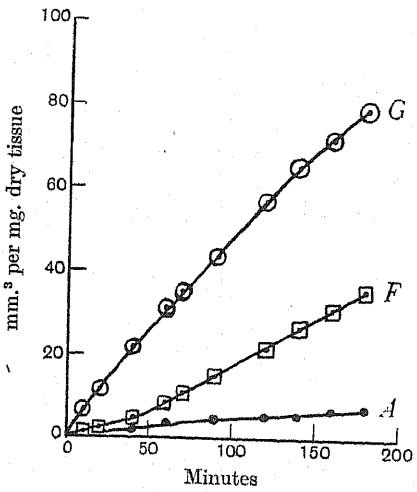


Fig. 1 (*a*). Jensen rat sarcoma.

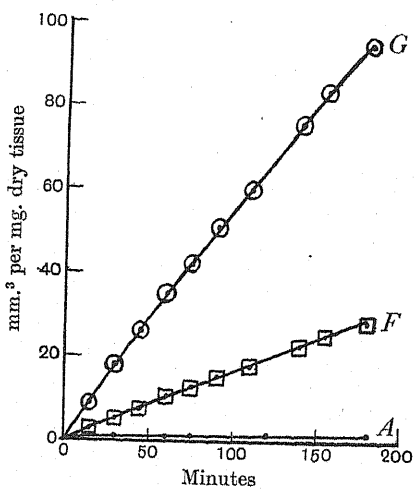


Fig. 1 (*b*). Mill Hill fibro-sarcoma I.

G, Glucolysis; *F*, fructolysis; *A*, autoglycolysis.

average of 12 experiments on different tumours $Q_M^{N_2}$ in the period before the rise was 8·2, and after the rise the average steady value was 12·2. Whilst the latter value is about 30 % higher than Rosenthal's average, the average increase of 49 % found by us is in fair agreement with his figure. Since a sudden increase in the rate of acid production in manometric experiments

may sometimes by due to a growth of contaminating bacteria, and since, when asepsis was not observed in the preparation of the tissue, the fructose solutions after removal from the vessels were almost invariably found to be contaminated with *B. welchii*, experiments on two tumours were carried out with aseptic precautions, in which sterility after the experiment was proved by the complete absence of growth overnight in anaerobic tubes. In both experiments an increase in the fructolysis occurred 50 minutes after the vessels were put in the bath, the increases being 30 and 36 %. As these are quite normal values, it appears that the usual contamination has no effect in these 3-hour experiments. Some simultaneous values of the glucolysis, fructolysis and autoglycolysis of Jensen rat sarcomas are given in Table II, the relative fructolysis for the second hour being given in the last column.

Table II. *Tumour tissues* $Q_M^{N_2}$.

Date	Glucolysis 1st hr.	Glucolysis 2nd hr.	Fructolysis 1st hr.	Fructolysis 2nd hr.	Autoglycolysis 1st hr.	Autoglycolysis 2nd hr.	Relative fructolysis (2nd hr.) %
Jensen rat sarcoma:							
15. ii. 32	26·0	23·0	8·0	12·0	3·0	2·0	48
3. iii. 32	33·0	23·0	8·9	10·0	3·8	1·7	39
4. iv. 32	36·0	31·0	9·8	10·2	6·7	2·2	28
5. iv. 32	30·0	24·5	9·1	9·5	2·5	1·7	
			10·4	10·8	—	—	35
			8·0	8·7	—	—	
30. vi. 32	31·0	27·0	8·9	12·7	2·6	2·3	42
26. vii. 32	36·5	34·0	11·0	15·5	3·1	2·0	42
Mean values	**32·1**	**27·1**	**9·3**	**11·2**	**3·6**	**2·0**	**39**
Mouse carcinoma 63:							
25. vii. 32	12·8	11·2	1·9	0·6	4·9	1·1	0
26. vii. 32	14·4	12·8	2·8	1·2	3·2	1·4	0
			(0·6 %) 4·8	2·4	—	—	9
Mouse glycogen carcinoma 113:							
25. vii. 32	24·7	24·3	2·5	1·3	2·0	0·7	3
	17·8	17·4	2·3	1·3	3·1	1·2	0
26. vii. 32	7·6	7·6	1·4	0·7	1·1	0·4	4
			(0·6 %) 3·8	1·0	—	—	8
Mouse sarcoma 3187:							
25. vii. 32	29·5	25·5	4·0	1·2	3·0	1·2	0
26. vii. 32	38·0	33·0	7·5	2·5	4·7	0·8	5
			3·7	1·3	—	—	2
Brown-Pearce tumour:							
7. iv. 32	16·5	—	2·0	—	1·8	—	(0)
18. iii. 32	10·5	9·0	2·8	1·1	2·2	1·4	0
			3·9	1·7	—	—	—
Rous chicken sarcoma:							
24. ii. 32	21·0	19·0	14·5	9·5	8·0	1·5	46
22. iii. 32	—	—	7·5	5·5	3·7	1·1	—
			5·4	3·6	—	—	—
31. iii. 32	53·0	41·0	10·0	4·0	4·0	0·5	9
Mill Hill fowl fibro-sarcoma No. 1:							
19. iii. 32	20·0	18·0	8·5	6·5	2·0	1·0	32
			9·0	6·5	—	—	32
21. iii. 32	34·0	32·0	10·5	9·5	1·0	0·5	29
			10·5	9·5	—	—	29
15. vii. 32	33·5	27·5	12·9	10·1	4·0	2·1	32
	41·2	—	17·3	—	—	—	—

Mouse tumours. Simultaneous measurements of the glucolysis, fructolysis and autoglycolysis of three different strains of mouse tumour are given in Table II. There is a great variability in metabolism of different mouse tumours of the same strain [Crabtree, 1929] which is reflected in the glucolysis figures given by us. It will be seen that none of the three mouse tumours attacks fructose anaerobically, even when the fructose concentration is raised to 0·6 %, for the differences between the fructolysis and autoglycolysis figures are in all cases of the same order of magnitude as the differences that we should expect to find between the autoglycolysis figures for different slices of the same tumour. It is interesting to note that the glycogen carcinoma does not fructolyse, for the two adult normal tissues which have large glycogen stores, namely muscle and liver, are the only ones which readily convert fructose into lactic acid, and it might have been supposed that some connection exists between fructolysis and glycogen-storage.

Rabbit tumour. Fructose is not converted into lactic acid by the Brown-Pearce rabbit tumour (Table II).

Fowl tumours. The Rous sarcoma provides very unsuitable material for metabolism experiments, as the material is heterogeneous and the recovery at the end of the experiment is difficult [*cf.* Crabtree, 1928]. It seems probable, however, from the figures in Table II, that this tumour can attack fructose anaerobically. Much better material is provided by the Mill Hill fowl fibro-sarcoma No. 1, which shows a steady anaerobic fructolysis for at least 3 hours (Fig. 1 (*b*)). We have obtained no evidence that the spontaneous increase of fructolysis, which is observed with the Jensen sarcoma, occurs also in the Mill Hill tumour, even though the glucolysis of the two tumours is about the same, and the steady fructolysis of the Mill Hill tumour is of the same order of magnitude as the final steady fructolysis of the Jensen sarcoma. The relative fructolysis of the Mill Hill tumour in the second hour is about 30 %, thus differing little from that of the Jensen sarcoma.

The lactic acid formed by the Mill Hill tumour was estimated by Clausen's method, in the way previously described [Dickens and Greville, 1932, 1]. The results show the acid production in fructose to be a pure lactic acid fermentation. For example, in one experiment the preformed and total quantities of lactic acid estimated chemically were 0·22 and 1·07 mg. respectively, so that the lactic acid formed in the experimental period of 180 minutes was 0·85 mg. The simultaneous manometric readings gave 0·87 mg. of lactic acid.

Summarising the results obtained with tumours, we see that the ability to fructolyse is not possessed by all tumours, and that the different types of tumour—sarcoma and carcinoma alike—show great differences in their behaviour towards fructose.

Factors influencing fructolysis.

The effects of the addition of sodium fluoride, and of alterations of the fructose concentration, upon the anaerobic fructolysis of Jensen rat sarcoma have been studied, in order to obtain evidence as to whether the conversion of fructose into lactic acid is a similar process to the conversion of glucose into lactic acid by this tumour.

Effect of sodium fluoride. As the anaerobic fructolysis increases spontaneously in the first hour of the experiment, all measurements were made in the second hour, the fluoride being added after the vessels had been one hour in the thermostat and the spontaneous increases had occurred. Since it was necessary to use calcium-free Ringer solution, however, these increases were somewhat masked by the slight falling off in $Q_M^{N_2}$ which occurs in a prolonged anaerobiosis in the absence of calcium ions. The effect of fluoride on the second-hour glucolysis of the same tissue was simultaneously determined in the same way. The inhibitions produced by four different fluoride concentrations are shown in Fig. 2, in which a curve showing the effect of fluoride on the glucolysis of rat testis in the first hour [Dickens and Šimer, 1929] is given for comparison.

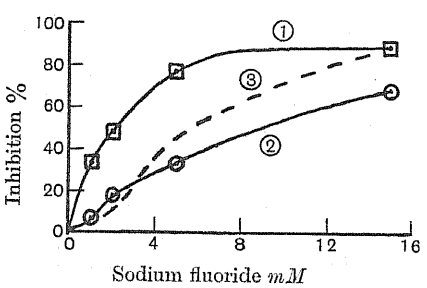


Fig. 2.

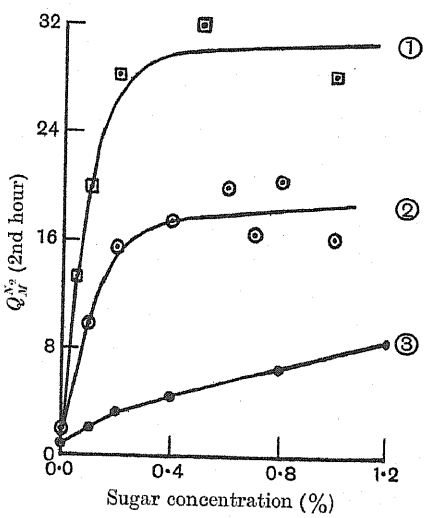


Fig. 3.

Fig. 2. (1) Jensen rat sarcoma in glucose (2nd hour).
(2) Jensen rat sarcoma in fructose (2nd hour).
(3) Testis in glucose (1st hour).

Fig. 3. (1) Jensen rat sarcoma in glucose.
(2) Jensen rat sarcoma in fructose.
(3) Rat liver in fructose [Rosenthal, 1931].

It appears from the observations of Dickens and Šimer [1929; 1930] that in the tissues examined the sensitiveness of the anaerobic glucolysis to fluoride runs roughly parallel with the magnitude of the anaerobic glucolysis in the

absence of inhibitor. The concentration of sodium fluoride necessary to produce 50 % inhibition of glycolysis for various tissues is given below together with the uninhibited glycolysis measured under the same experimental conditions; and it will be seen that the effect of fluoride on the anaerobic fructolysis of Jensen rat sarcoma in the second hour fits quite normally into the series. The effect of fluoride therefore does not suggest that in its conversion into lactic acid by the Jensen tumour, fructose proceeds by a route very different from that pursued by glucose.

Tissue	Substrate	Hour of exp.	$Q_M^{N_2}$	Concentration of NaF causing 50 % inhibition (mM)
Retina	Glucose	1st	60	<1
Jensen sarcoma	,,	1st	22	1·3
Jensen sarcoma	,,	2nd	21	2·0
*Chick embryo (4th day)	,,	1st	17	6·0
Testis	,,	1st	7·3	6·0
Jensen sarcoma	Fructose	2nd	6·7	9·0
Kidney	Glucose	1st	3	10·0

* Dickens and Greville (hitherto unpublished observation).

Fructose concentration. The anaerobic glycolysis of Jensen rat sarcoma in the second hour in glucose and in fructose was measured in Ringer solutions of different sugar concentrations, and the results of typical experiments are shown in Fig. 3. The velocity-sugar concentration curve for glucose agrees well with that given for Flexner-Jobling rat carcinoma by Warburg *et al.* [1924], and it will be seen that the glucose and fructose curves are similar in shape. In both cases the glycolysis does not increase much when the sugar concentration is raised above 0·2 %, and in both cases the substrate concentration at which half the limiting velocity is reached lies near 0·1 %. A curve showing the influence of fructose concentration on the anaerobic fructolysis of rat-liver in the second hour is also given for the sake of comparison in Fig. 3, the figures being taken from Rosenthal [1931]. The form of this curve is quite different from those of the Jensen rat sarcoma; the possible significance of this will be discussed later.

Spontaneous increase in fructolysis. We have made some observations on the occurrence of the spontaneous increase in fructolysis which happens with Jensen rat sarcoma during the first hour of the experiment. In these experiments the values of $Q_M^{N_2}$ in periods before and after the rise are denoted by A and B respectively, and the magnitude of the rise is expressed as a percentage by the quotient $100\ (B - A)/A$ [Rosenthal, 1930, 2]. The spontaneous increase occurs in the presence of phosphate, for in a Ringer solution containing both phosphate and bicarbonate an increase of 35 % was found when the necessary correction was made for carbon dioxide retention. In pure bicarbonate-Ringer, it was found from the experiments with varying fructose concentrations that the spontaneous rise is maximum when the fructose concentration is about 0·2 %; in 1 % fructose the increase is negligible. The

percentage increases with different sugar concentrations found in three different experiments are given below:

Fructose concentration %	Spontaneous increase of fructolysis (%) $\frac{100(B-A)}{A}$ (1)	(2)	(3)
0·1	+35	+27	− 2·5
0·2	+44	+48	+50
0·4	.	+13	+45
0·5	+ 7	.	.
0·6	.	+15	+20
0·8	.	− 2·5	.
1·0	+ 3	+ 9	.

The effect of the addition of sodium pyruvate on the spontaneous increase observed in Jensen rat sarcoma has already been reported [Dickens and Greville, 1932, 2], and is discussed below.

Discussion.

The spontaneous increase in fructolysis observed in Jensen rat sarcoma was ascribed by Rosenthal [1930, 2] to the anaerobic formation and accumulation of an activator. This spontaneous activation, he considers, is analogous to the activation produced in liver tissue by a preliminary aerobiosis. Various differences, however, exist between the activation phenomena in the two tissues; the most important of these are as follows. Firstly, activation occurs anaerobically in tumour tissue, but only under aerobic conditions in liver. Secondly, the activated glycolysis falls off rapidly in liver, but continues practically constant in tumour. Lastly, whilst activation occurs in liver in the presence of any fermentable sugar, the activation phenomenon in tumour is readily seen only in the fructose metabolism. However, Rosenthal considers that these differences do not necessarily mean that the activation process is not similar in the two tissues, and that "soweit sich Abweichungen zeigten, konnten sie in ihrer Mehrzahl durch Unterschiede der verglichenen Gewebe im Gehalt an Aktivator bzw. zu seiner Bildung nötigen Substanzen und in der Geschwindigkeit der Aktivatorbildung erklärt werden." Some observations made by us [Dickens and Greville, 1932, 2] have led us to agree that the activation phenomena in liver and tumour are analogous. We have been able directly to demonstrate the spontaneous activation of the glucolysis in Jensen sarcoma, which Rosenthal had concluded must occur [Rosenthal, 1930, 2, footnote, p. 237]. Again, we found that the addition of 10^{-3} M sodium pyruvate to the Jensen rat sarcoma in fructose before the spontaneous increase had occurred caused an immediate increase in fructolysis, $Q_M^{N_2}$ thereafter remaining constant at about the value it would have reached by spontaneous activation, whilst addition of the pyruvate after the spontaneous increase had occurred had no effect at all on the fructolysis. Since Rosenthal [1932] has shown that sodium pyruvate produces an activation of the anaerobic

autoglycolysis and fructolysis of liver similar to that produced by preliminary aerobiosis, it certainly seems that activation in liver and tumour are similar processes. Rosenthal considers that in liver sodium pyruvate is not the activator; but whether in tumour tissue pyruvate can replace the activator, or is an intermediate substance in the chain of reactions whereby the activator is formed, or is even the activator itself, cannot possibly be decided from our present knowledge.

In the Mill Hill sarcoma we have been unable to demonstrate spontaneous activation of the fructolysis, and here pyruvate had no effect [Dickens and Greville, 1932, 2]. It may be that activation is extremely rapid, but as the rate of activation, according to Rosenthal, depends on the magnitude of the glycolysis, and the fructolysis of the two tumours is about the same, this seems unlikely. The possibilities remain that de-activation does not occur, or that the mechanism for spontaneous activation, even when pyruvate is supplied, is missing. We are not in a position to decide between these possibilities and explain why the Mill Hill and Jensen tumours behave so differently. Indeed, as we cannot explain why increase of fructose concentration, as shown earlier, reduces the apparent magnitude of the spontaneous increase, why activation occurs anaerobically in tumour but only aerobically in liver, and why the glycolysis falls off rapidly in liver, although this is prevented by increasing the sugar concentration, it is clear that much more work must be done before the activation phenomena become at all intelligible.

It does not seem improbable that in the Jensen sarcoma glucose and fructose are fermented by similar routes. In yeast it has been suggested that because of the uniformity of rate of fermentation of glucose and fructose, these sugars are probably fermented by the same enzyme, glucozymase [see Harden, 1932]. This possibility is strengthened by Dawson's demonstration [Dawson, 1932] that the phenomena of selective fermentation of glucose and fructose by living yeast may be explained on Michaelis's theory of enzyme affinity by the competition of substrates. In Jensen rat sarcoma the normal effect of fluoride found above and the observation of Rosenthal [1930, 2] that glyceraldehyde completely inhibits both fructolysis and glucolysis, suggest that the fermentation paths coincide at some stage between hexose and methylglyoxal, whilst the glycolysis-sugar concentration curves for the two sugars are similar to those which would be obtained if the fructose and glucose conversions were catalysed by the same or similar enzymes, and glucose had two or three times the affinity of fructose for the enzyme.

With the fructolysis of liver the situation is different and very complicated. The metabolism of surviving liver shows several striking anomalies. Liver is the only tissue known to be unable to convert glucose into lactic acid. It differs from the other adult normal tissues (except muscle) in converting fructose into lactic acid, and from tumours in its ability to glycolyse glyceraldehyde [Rosenthal, 1930, 2]. The extraordinary dependence of both aerobic and anaerobic metabolism on the state of nutrition, of the fructolysis on the time

of year, and of the anaerobic metabolism in general upon the presence of an activator which may be produced by preliminary aerobiosis [Rosenthal, 1929] or the addition of pyruvate, acetaldehyde or methylene blue [Rosenthal, 1932], shows that many different factors are operating to determine the glycolysis of any sample of liver tissue. It would therefore be dangerous to draw any definite conclusions from the fact that the fructolysis increases with the fructose concentration in quite an unusual way (Fig. 3). A possible explanation might be that increased fructose concentration, since it prevents the rapid fall of glycolysis with time, produces greater amounts of activator; but it seems to us that the fall of glycolysis may be due to causes other than the loss of activator. For not only does it occur both in the autoglycolysis and fructolysis, but it occurs equally in the activated glycolysis produced both by pyruvate and preliminary aerobiosis [Rosenthal, 1931; 1932]; moreover, after the falling off, Rosenthal [1931] could not restore the activation by a further aerobiosis. If, then, the effect of raising the sugar concentration does not produce activator, it may be that fructose has a very low affinity for the enzyme, so that a velocity equal to half the limiting velocity is reached only at a relatively very high substrate concentration. There is, however, one other possible explanation which we think should be borne in mind. In view of the fact that glucose is not glycolysed by liver, it is possible that the substrate of liver fructolysis is not the ordinary form *n*-fructose, but is some labile *h*-fructose which is in equilibrium with it in aqueous solution. Then the fructose concentration would have to be raised very considerably before the enzyme system became saturated with this labile sugar. Whatever the explanation, however, it must be conceded that liver fructolysis is of a type very different from the usual glycolysis.

Meyerhof's view, that the fructolysis of a tissue depends on the existence in that tissue of an initial phosphorylation stage, was discussed in the previous paper, and therefore no further reference to it is necessary here. It appears from the present paper that variations in the ability to fructolyse occur just as strikingly among the tumour tissues as among the embryonic tissues. The appearance of fructolysis in one tissue and its complete absence from another tissue of similar type, the great variability shown by the various tissues in their attack on fructose when their metabolism of glucose is similar, still await an explanation, which, when it is forthcoming, can hardly fail to clear up many obscure features of carbohydrate metabolism.

Summary.

1. The anaerobic conversion of fructose into lactic acid by tumour and adult normal tissues has been studied.

2. The anaerobic attack of rat's retina, submaxillary and spleen on fructose is negligible, whilst kidney and testis show a slight attack. When these results are taken in conjunction with those obtained by other authors on brain, nerve

and liver, it is seen that those adult normal tissues which convert glucose into lactic acid show little anaerobic attack on fructose, whilst liver, which cannot utilise glucose anaerobically, can under certain conditions readily form lactic acid from fructose.

3. The Jensen rat sarcoma and the Mill Hill fowl fibro-sarcoma No. 1 show a steady anaerobic fructolysis equal to about a third of the glucolysis. The Rous chicken sarcoma may also show some attack on fructose, but the other types of tumour examined (the Brown-Pearce rabbit tumour, two mouse carcinomas and one mouse sarcoma) lack the ability to convert fructose into lactic acid. There thus exists among the tumour tissues a variability in their behaviour towards fructose which is as pronounced as that found among the embryonic tissues.

4. The spontaneous increase in anaerobic fructolysis observed by Rosenthal in the Jensen rat sarcoma has not been observed in the Mill Hill tumour. Rosenthal's activator theory is discussed.

5. The effects of the addition of sodium fluoride and of alteration of substrate concentration upon the anaerobic fructolysis of Jensen rat sarcoma have been determined, and the possibility that glucose and fructose are converted into lactic acid by similar routes in this tumour is discussed.

We wish to express our thanks to Prof. E. C. Dodds for his interest in these experiments, and to Dr L. E. H. Whitby for the bacteriological controls he has carried out for us. We are indebted to the following for kindly supplying us with material; Dr J. A. Murray, of the Imperial Cancer Research Fund, for mouse tumours; Prof. J. McIntosh and Prof. S. Russ, of this hospital, the former for fowl and rabbit tumours, the latter for rat sarcomas.

One of us (F. D.) is a member of the Scientific Staff of the Medical Research Council, and the other (G. D. G.) wishes to make acknowledgment to the Halley Stewart Trust Fund for a scholarship.

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CLXXXV. THE DENATURATION OF PROTEINS.

IX. THE EFFECT OF THE ADDITION OF ELECTROLYTES ON THE VISCOSITY OF SOLUTIONS OF CRYSTALLINE EGG-ALBUMIN.

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In a recent communication, Loughlin and Lewis [1932] examined the effect of denaturation on the viscosity of solutions of crystalline egg-albumin and oxyhaemoglobin, and found that in each case the process of denaturation is accompanied by an increase in viscosity. Since dilatometer measurements show that there is no change in the total volume of the protein solutions on denaturation, the above observation was interpreted to mean that the process of denaturation involves a physical distension of the protein unit accompanied by an increase in the amount of water associated with each protein particle, this conclusion being in accordance with the well-known fact that denaturation will not occur in the absence of water.

It was found that for a given concentration of undenatured protein solution the viscosity was a constant independent of p_H, as was also the viscosity of a given concentration of denatured protein solution. This was unexpected in view of the theory of Smoluchowski [1916] to the effect that when the particles in a solution are electrically charged the viscosity of the solution should be increased as a result of the charges. Alteration of the p_H from the isoelectric point necessarily involves a considerable alteration in the electric charge of the protein units, and thus appears to create conditions in which an electro-viscous effect would be anticipated. Furthermore this electro-viscous effect would be expected to increase as we recede from the isoelectric point.

Consequently in view of the uncertainty which necessarily existed as to the interpretation of the viscosity results at p_H values removed from the isoelectric point, it was deemed advisable, in the paper referred to, to interpret the results in terms of particle size at the isoelectric point alone, pending a further series of determinations of viscosity and conductivity which would settle whether or not an electro-viscous effect had to be taken into account. The results of these further determinations are given in the present communication, and from them a general conclusion is drawn concerning the mechanism of the process of denaturation at all p_H values.

Experimental.

The protein used was crystalline egg-albumin prepared by the method of Sørensen [1917], and then freed from salt by prolonged dialysis. The concentration of protein in the stock solution was estimated by the method of Devoto as modified by Hopkins [1900].

The viscosities were measured by the times of outflow through the capillary tube of an Ostwald viscosimeter, the "relative viscosity" given being the ratio of the time of outflow of a certain volume of protein solution to the time of outflow of the same volume of pure water through the same viscosimeter at the same temperature. The instrument was tested as described [1932] to ensure that the viscosities measured were true viscosities, and in all cases the mean value of at least five readings was taken. Other experimental details are as before.

The p_H determinations were made at a temperature of 18° by the method of Harrison [1930] using the glass electrode.

The conductivities were measured using the ordinary type of conductivity cell, the resistance of the cell in each case being obtained by an adaptation of the Wheatstone bridge method. The cell constant was obtained by measuring the resistance of the cell when it contained $N/50$ KCl. All conductivity measurements were made in a thermostat at a temperature of 25°. In all cases the resistance of the cell was measured against a series of about ten resistances in the resistance box of the Wheatstone bridge, and two samples of each solution were used. Thus each conductivity measurement given in this paper is a mean value of about twenty readings.

The viscosity and conductivity of 3 % salt-free solutions of undenatured crystalline egg-albumin at 25°.

The 3 % solutions over the required p_H range were prepared from the stock solution as described in the previous paper. The viscosity was then measured at 25° exactly as before. The electrodes in the conductivity cell were well washed with the albumin solution of which 10 cc. were measured into the conductivity vessel. The specific conductivity was then determined as described above. The cell was emptied, and a new 10 cc. sample of the albumin solution was introduced. Once again the specific conductivity was measured and the mean value of all the readings was finally recorded.

In this way the viscosity and conductivity of a series of solutions, ranging from the isoelectric point down to p_H 3·0 were measured, the readings being given below.

Mean time of flow for water = 131·8 secs.

p_H	Time of flow (seconds)	Relative viscosity	Spec. conductivity $\times 10^4$ (mhos)
3·01	152·5	1·157	66·1
3·29	152·9	1·160	32·3
3·88	153·0	1·161	10·0
4·34	152·4	1·156	4·4

Mean value of the relative viscosity = 1·158.

It is thus seen that although the sulphuric acid required to reduce the p_H from the isoelectric point down to 3·0 causes a large increase in the conductivity, there is no appreciable effect on the viscosity. The significance of this will be pointed out later.

There was no necessity to perform a similar series of experiments, either with denatured egg-albumin or with oxyhaemoglobin, since previous viscosity measurements have shown that constant values of the viscosity were obtained for any given solution irrespective of its p_H. Since the p_H was adjusted with sulphuric acid a similar increase in conductivity on either side of the isoelectric point would necessarily be experienced. Consequently the one series of quantitative results suffices for our present purpose.

The viscosity and conductivity of 3 % solutions of undenatured egg-albumin containing sodium chloride.

It has been shown that an alteration in the conductivity of a given albumin solution by means of sulphuric acid does not affect the viscosity. In view of this finding, an alteration in conductivity produced in any other way, *e.g.* by the addition of a neutral salt, should not affect the viscosity, but as a greater range of conductivity could be covered by this means, it was thought worth while to carry out a series of experiments in the presence of sodium chloride. Furthermore, assuming for the moment that Smoluchowski's view is the correct one, the greater the change in conductivity produced, the greater should be the deviation in viscosity due to the electro-viscous effect. Similar measurements for denatured egg-albumin could not be made as the addition of salt causes flocculation.

The experimental details were exactly as above, excepting that each 3 % solution was made $N/100$ with respect to sodium chloride by addition of the requisite amount of sodium chloride solution in the process of dilution of the stock albumin solution down to 3 %. As before, the p_H was adjusted with sulphuric acid.

3 % solutions of undenatured egg-albumin containing 0·01 N sodium chloride at 25°.

Mean time of flow for water = 131·8 seconds.

p_H	Time of flow (seconds)	Relative viscosity	Spec. conductivity $\times 10^4$ (mhos)
2·31	153·1	1·161	105·4
3·46	153·3	1·163	79·4
4·18	153·3	1·163	26·4
5·30	152·9	1·160	12·2

Mean value of the relative viscosity = 1·162.

Thus once again the viscosity is found to be a constant independent of p_H, although the conductivity increases rapidly as the p_H is removed from the isoelectric point. The mean value of the viscosity is 1·162 which is slightly greater than the value 1·158 for the salt-free solutions, the difference however,

less than 0·4 %, being within the limits of experimental error. Experiment shows that the viscosity of $N/100$ NaCl is not materially different from that of pure water.

Although examination of the available work shows that a concentration of 10 milli-equiv. of electrolyte is quite sufficient to produce the maximum depression of the viscosity of those sols for which an electro-viscous effect has been observed, it was deemed advisable to make one or two measurements of viscosity and conductivity with a much higher electrolyte concentration, *viz.* 100 milli-equiv. per litre. It should be noted that albumin solutions are quite stable at this concentration of neutral salt, and that the process of denaturation, although accelerated, has been shown by Lewis [1926] to be still unimolecular, and quite normal in all respects in the presence of even as high concentrations of sodium chloride as 3·4 *N*.

3 % solutions of undenatured egg-albumin containing 0·1 N *sodium chloride at 25°.*

p_H	Time of flow (seconds)	Relative viscosity	Spec. conductivity $\times 10^4$ (mhos)
2·40	153·8	1·167	121·4
4·00	151·6	1·150	104·1

Mean value of the relative viscosity = 1·159.

From this it is seen that even when the concentration of sodium chloride is increased to 0·1 *N* there is still no appreciable effect on the viscosity.

Discussion.

According to Smoluchowski [1916], when the particles in a solution are electrically charged, the viscosity of the solution is increased as a result of the charges, the value of the viscosity being given by the equation

$$\eta = \eta_0 \left[1 + 2{\cdot}5\,\phi_E \left\{ 1 + \frac{\sigma}{a^2 \eta_0} \left(\frac{\kappa\zeta}{2\pi} \right)^2 \right\} \right],$$

where σ is the specific resistance of the solution, a is the particle radius, ζ is the electrokinetic potential difference at the interface particle-medium, κ is the dielectric constant of the medium, and η_0 its viscosity. It will be noticed that at the isoelectric point, *i.e.* when ζ is zero, the formula becomes the simple Einstein formula. Thus the ϕ value is that which appears in the Einstein equation, and it is accordingly represented as ϕ_E.

On Smoluchowski's formulation, therefore, the electro-viscous term is expressed as a correction factor of the form $(1 + x)$ multiplied on the term ϕ which stands for the fraction of the total volume of the system occupied by the disperse phase. The factor x is given the value

$$\frac{\sigma}{a^2 \eta_0} \left(\frac{\kappa\zeta}{2\pi} \right)^2 \quad \text{or} \quad \frac{4\sigma\eta_0 v_0^2}{a^2},$$

where v_0 is the electrophoretic velocity of the particles under unit electrostatic potential gradient, and the other symbols have the same significance as before.

It follows from the above expression that an increase in ζ (due to increase in surface charge) should increase the viscosity, whilst on the other hand, any reduction in ζ (say by the addition of salt) should decrease the viscosity.

Clearly Smoluchowski had in mind the case of a lyophobic system in which the radius a likewise determines the value of ϕ. In the case of proteins we are dealing in general with essentially hydrated structures. In this case the term a must stand for the radius of the ionisable protein kernel upon the surface of which are located electric charges whose lines of force pass outwards through the surrounding shell of water of hydration. On the other hand, the volume occupied by the disperse phase will depend not only on a but also upon the degree of hydration, and upon whether this varies with p_H or not. In view of this, let us consider very briefly the attempts that have been made from time to time to verify Smoluchowski's equation for various systems.

By far the most work has been performed on sols of agar, gum arabic, and starch, and to a less extent on colloidal hydroxides, sulphides and oxides, and on certain dyes. Very little attention has been paid to proteins, and none at all to the two proteins with which we are dealing in the present paper.

Kruyt and de Jong [1928] state that all electrolytes diminish the viscosity of agar sols even in small concentrations. Salts containing univalent cations in equivalent concentrations cause practically the same decrease. Salts containing bivalent cations in equivalent concentrations also cause equal decreases in the viscosity, the magnitude of the decrease being greater than in the previous case. The decrease in viscosity in fact is found to depend chiefly on the valency of the cation of the added electrolyte. It may be added that cataphoretic velocity measurements showed that the agar sols are negatively charged. The conclusion drawn from these experimental results is that the Smoluchowski formula cannot be applied to agar sols quantitatively, but it can be applied qualitatively.

Sols of soluble starch were observed by de Jong [1924] to act towards electrolytes exactly as did the agar sols above. Kruyt and Tendeloo [1929, 1] observed the same effects with gum arabic.

Dhar and Ghosh [1929] examined more than thirty sols of the colloidal metallic hydroxide, sulphide, *etc.* types, and found that addition of electrolyte lowered the viscosity.

In the case of proteins, Pauli and Handovsky [1908; 1910] state that the viscosity of ox-serum is diminished by the addition of neutral salts. Kruyt and Tendeloo [1929, 2] state that gelatin and caseinogen act in a manner similar to starch and gum arabic. No observations appear to have been made on egg-albumin or oxyhaemoglobin.

In addition to the investigations referred to above, wherein it is concluded that the Smoluchowski equation gives a qualitative idea of the behaviour to be expected when salts are added to the sols mentioned, a few attempts at a quantitative application have likewise been made.

Thus Kruyt and Tendeloo [1924] using 0·5 % sols of starch containing

various concentrations of different electrolytes measured in each case the viscosity, the specific conductivity, and the cataphoretic velocity. By substituting the values obtained in the Smoluchowski equation a number of equations involving the two unknowns ϕ and a were thus obtained. These equations were solved in pairs and values of ϕ and a obtained. The values of ϕ varied from 4·8 to 5·8 $\times$ 10^{-2} and a varied from 5·4 to 9·7 $\times$ 10^{-7} cm. Such calculations imply, however, that ϕ and a do not themselves vary with the charge of the sol, which is continually modified by the addition of electrolyte. The authors conclude that constant values of ϕ and a are actually obtained, thus confirming the supposition which was made in the first place.

Tendeloo [1927] measured the viscosities, specific conductivities and cataphoretic velocities of arsenious sulphide sols containing various amounts of electrolytes, and obtaining the value of ϕ from the weight of arsenious sulphide in the sol by assuming its density to be unaltered in solution, he was able to obtain, with the aid of the equation of Smoluchowski, a number of values of a. These ranged from 5 $m\mu$ to 7·4 $m\mu$ giving a mean value of 6 $m\mu$. Tendeloo considers that the constancy of the value for a may be regarded as an indication that the Smoluchowski equation is applicable to the arsenious sulphide sols.

Finally Prideaux and Howitt [1932] found that the viscosities of agar sols between 2 and 7 % are much greater than the values calculated from the equation of Smoluchowski when electrolyte is present. They mention that, strictly speaking, this formula applies only to suspensoids, the solution volume ϕ and radius a of which are known, but that it can also be used in a semi-quantitative manner for lyophilic colloids.

Considering the above work on the electro-viscous effect, it is evident that the Smoluchowski equation has not been verified as yet in an entirely satisfactory manner, although it leads in the cases of starch and arsenious sulphide sols to values of a which are well within the bounds of possibility.

It is significant, however, that practically all the sols upon which work has been performed are of such a nature that their charge is due to the adsorption of ions, and not to structural ionisation of the colloidal unit itself. Consequently the effects to be observed in such cases are of a totally different nature from those which one would expect for egg-albumin and oxyhaemoglobin, and the above work can be of little help in connection with the question as to whether the Smoluchowski equation applies to protein solutions or not.

As we have seen, the striking experimental fact is that the viscosities of egg-albumin and oxyhaemoglobin solutions are constant and independent of p_H, although alteration in p_H from the isoelectric point necessarily involves a considerable alteration in the electric charge of the protein units. How then can this be reconciled with the view of Smoluchowski?

One explanation which immediately suggests itself is that the Smoluchowski correction does in fact exist, but is so small that its effect on the viscosity is insufficient to be observed experimentally. This possibility is immediately disposed of, however, if we take some definite p_H removed from the isoelectric

point, and calculate the value of the correction. Let us take for example 3 % salt-free egg-albumin solution at p_H 3·0. As mentioned above, the correction factor x is given by the expression

$$x = \frac{4\eta_0 v_0^2 \sigma}{a^2} = \frac{4\eta_0 v_0^2}{\kappa a^2} \text{ where } \sigma = \frac{1}{\kappa}.$$

The value of η_0 given in the International Critical Tables is 0·00894 poises, v_0 from the cataphoretic velocity measurements of Scott and Svedberg [1924] is $21 \times 10^{-5} \times 300$ cm. per sec., κ from the writer's experiments is 66×10^{-4} mhos or 6×10^9 electrostatic units, and a is of the order 2×10^{-7} cm., therefore

$$x = \frac{4 \times 0{\cdot}00894 \times (21 \times 10^{-5} \times 300)^2}{6 \times 10^9 \times (2 \times 10^{-7})^2} = 0{\cdot}6,$$

which is by no means negligible compared with unity. This means that the equation of Smoluchowski would predict an increase of 60 % in the ratio $\frac{\eta - \eta_0}{\eta_0}$ for a 3 % albumin solution when the p_H is altered from the isoelectric point down to p_H 3·0. Such a large increase in viscosity, needless to say, would readily be observed experimentally. Thus the suggestion that the electroviscous effect is negligible is untenable. At the same time the magnitude of the effect which the application of the formula would lead us to expect is unbelievably large. Such a marked increase in viscosity on the addition of a small amount of sulphuric acid indeed seems improbable. The constancy of viscosity with p_H is therefore in direct conflict with the theory of Smoluchowski.

Let us next consider whether the experiments on the protein solutions containing neutral salt are also in conflict with the behaviour one would expect on this theory. Once again we will take the case of a 3 % egg-albumin solution at p_H 3·0, but this time in the presence of $N/100$ sodium chloride. The values of η_0 and a are the same as in the previous case, and v_0 is also unaltered, as examination of the cataphoretic velocity measurements of Abramson [1928], Svedberg and Tiselius [1926], and Scott and Svedberg [1924] shows that the cataphoretic velocity is the same at different concentrations of albumin, and also in the presence of different buffer solutions, *i.e.* in the presence of different concentrations of different salts. The value of κ from the table given on p. 1559 is 90×10^{-4} mhos or $8{\cdot}1 \times 10^9$ electrostatic units. Thus

$$x = \frac{4 \times 0{\cdot}00894 \times (21 \times 10^{-5} \times 300)^2}{8{\cdot}1 \times 10^9 \times (2 \times 10^{-7})^2} = 0{\cdot}44.$$

This means that the presence of $0{\cdot}01N$ sodium chloride at p_H 3·0 should cause the value of the ratio $\frac{\eta - \eta_0}{\eta_0}$ to decrease to $\frac{1{\cdot}44}{1{\cdot}60}$ of its original value, *i.e.* a decrease of 10 %. The experiments have shown, however, that the two viscosities are identical within the limits of experimental error, so that once again Smoluchowski's theory is in conflict with the experimental results. The discrepancies are not limited to p_H 3·0 but are general at all p_H values other than the isoelectric point.

In view therefore of these considerations, we can only conclude that the assumption made by Smoluchowski in obtaining his equation, *viz.* that the charge on the particles in the solution causes an increase in the viscosity of the solution, does not apply in the present case. In other words, the viscosity is not affected by a change in charge which has its origin in the ionisation of the individual protein particles. This suggests that the Einstein type of formula is applicable to the protein solutions under consideration, not only at the isoelectric point but also at all other p_H values. In other words, the interpretation of the viscosity results as an indication of the mechanism of the process of denaturation—which was restricted in the previous communication to the isoelectric point—can be extended to include the whole range of p_H covered by the experiments.

It follows that at all p_H values the process of denaturation of egg-albumin and oxyhaemoglobin is accompanied by an increase in the individual protein units to about twice their normal volume, this increase being accompanied by an increase in the amount of water associated with each unit.

Summary.

1. Viscosity and electrical conductivity measurements have been made on a series of solutions of undenatured crystalline egg-albumin in the absence of salts, and likewise in solutions containing 0·01 N and 0·1 N sodium chloride.

2. The viscosity was found in all cases to be constant and independent of p_H, although the conductivity increased rapidly as the p_H of the solution was removed from the isoelectric point.

3. The Smoluchowski equation for the viscosity of solutions containing charged protein particles has been compared with the results obtained, and is shown to be in conflict with the experimental observations.

4. It is concluded that no electro-viscous term in the sense of Smoluchowski need be taken into account in the case of the proteins under consideration, and the simple Einstein type of equation applies at all values of p_H.

5. The conclusion drawn from the viscosity results (and restricted in a previous communication to the isoelectric point), to the effect that the process of denaturation involves a distension of each individual protein unit accompanied by an increase in the amount of water associated therewith, so that the volume of each is approximately doubled, is now extended to hold at all p_H values.

In conclusion, the writer would like to express his thanks to Prof. W. C. M. Lewis, under whose direction this work was carried out.

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CLXXXVI. THE FORMATION OF ENTEROKINASE FROM A PRECURSOR IN THE PANCREAS: A SUPPLEMENTARY NOTE.

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In a recent paper [1932] the writer presented data upon the rate of formation of enterokinase from its precursor in the pancreas. It was found that the observed rate conforms to the unimolecular expression and that it is a function of the hydrogen ion concentration of the medium, exhibiting a maximum at about p_H 6·90. Certain additional experiments which have been carried out are now described.

The critical increment of the process.

It was considered that a knowledge of the critical increment (or energy of activation) might be of value in discussing the mechanism of the process. This was therefore determined by measuring the rate of formation at 20° and at 30° and then substituting the observed values into the equation for the critical increment:

$$E = \frac{2{\cdot}303\, RT_1T_2}{(T_1 - T_2)} \log_{10} \frac{k_{T_1}}{k_{T_2}},$$

where k_{T_1} and k_{T_2} are the velocity constants at the temperatures T_1 and T_2 respectively.

The experimental details were as follows. The solution containing the precursor (neutralised elution from alumina adsorptions of glycerol extract of dried pancreas powder) was prepared in the way previously described by the writer. To 40 cc. of neutralised elution were added 10 cc. of phosphate buffer such that the p_H of the mixture was 6·9. Then 20 cc. of the buffered mixture were measured out into each of two glass bottles. One of these was then placed in a thermostat at 20° and the other in a thermostat at 30°, and after samples had been removed for the determination of the zero reading the bottles were stoppered and kept in their respective thermostats.

It has already been shown that the rate of formation may be described by the unimolecular expression:

$$k = \frac{2{\cdot}303}{t} \log_{10} \frac{a}{a - x},$$

where a is the initial concentration of precursor, and $(a - x)$ is the concentration of precursor after time t.

In the writer's previous paper it was also shown that a may be taken to be the maximum amount of enterokinase formed—*i.e.* the "total extent" reading, which is reached after about 35 hours at 30°. Therefore in the present experiments a was determined in this way and a value of $(a - x)$ was obtained by measuring the quantity of enterokinase formed after 18 hours and then subtracting this from the a value. This difference is a measure of the quantity of precursor remaining unchanged after 18 hours.

The quantity of enterokinase formed in each case was measured by the method described previously. Table I gives the results of two separate experiments. Blank and control readings have been subtracted and the increases in acidity recorded are therefore direct measures of the quantity of enterokinase formed.

Table I.

	Increases in acidity cc. 0·1 N NaOH		$\frac{a}{(a-x)}$	k (t in mins.)	E (calories)
	18 hours	Total extent			
Exp. 1					
Temp. 20°	0·54	0·92	$\frac{0·92}{0·38}$	$8·2 \times 10^{-4}$	9600
Temp. 30°	0·72		$\frac{0·92}{0·20}$	$1·4 \times 10^{-3}$	
Exp. 2					
Temp. 20°	0·55	0·96	$\frac{0·96}{0·41}$	$7·9 \times 10^{-4}$	9500
Temp. 30°	0·74		$\frac{0·96}{0·22}$	$1·36 \times 10^{-3}$	

The significance of a value for the critical increment of this order, *viz.* 9500 calories, is considered later.

Adsorption experiments.

The solution which has been used so far in this work for observing the formation of enterokinase from a precursor has been the neutralised elution from alumina adsorptions carried out upon a glycerol extract of fresh dried pancreas gland powder. A solution prepared in this way is known to contain the two enzymes aminopolypeptidase and dipeptidase. These may be separated by ferric hydroxide adsorptions at p_H 4 (see for example Waldschmidt-Leitz and Purr [1929]). The dipeptidase is adsorbed by the ferric hydroxide under these conditions, while the aminopolypeptidase remains in solution. In view of this it was decided to examine whether the precursor of enterokinase also exhibited any specific adsorptive behaviour towards ferric hydroxide.

The ferric hydroxide was prepared by the method of Willstätter, Kraut and Fremery [1924] as follows. Two solutions were first made up, (*a*) a solution of 50 g. of ferric ammonium sulphate in 100 cc. of water, (*b*) 400 cc. of an aqueous solution containing 5·4 g. NH_3 and 13·75 g. of ammonium sulphate. Solution (*b*) was stirred vigorously with a mechanical stirrer and solution (*a*) then added as quickly as possible. The stirring was continued for half an hour. At the

end of this time the ferric hydroxide was in the form of a uniform reddish-brown precipitate. This precipitate was washed by diluting with distilled water to 2 litres, allowing it to settle again, and then decanting. This was carried out twelve times in all. At the second and third decantations 0·5 cc. of concentrated ammonia (sp. gr. 0·88) was added to decompose any unchanged sulphate.

The gel-like ferric hydroxide remaining after the final decantation was used for the adsorption experiments, which were carried out in the following way.

Four alumina adsorptions were first carried out upon 20 cc. of glycerol extract in the way previously described by the writer. The alumina from these adsorptions was then eluted with 20 cc. of 0·05 *N* ammonia, the alumina centrifuged off and the clear elution then neutralised with *N* HCl.

The neutralised elution was then brought to p_H 4 by addition of 5 cc. of an acetate buffer (made up by mixing 17·6 cc. *N* acetic acid and 2·4 cc. of *N* sodium acetate). The solution was then treated with 5 cc. of the ferric hydroxide suspension, the mixture left for about 15 minutes, and the ferric hydroxide then centrifuged off. The mother-liquor from this first adsorption was then treated again with 5 cc. of the ferric hydroxide suspension, and the process repeated. The final mother-liquor from the centrifuging was neutralised with *N* ammonia. The ferric hydroxide precipitate was eluted with 25 cc. of a 1 % Na_2HPO_4 solution; the ferric hydroxide then centrifuged off and the elution neutralised with *N* acetic acid. Samples of the neutralised mother-liquor and the neutralised elution were then kept at 30° and the formation of enterokinase was measured in the usual way by taking initial readings and readings after 20 hours at this temperature. Table II shows the results of a number of experiments. These are separate and individual experiments—in each case the whole adsorption procedure was carried out on different original samples of glycerol extract. Blank and control readings have been subtracted.

Table II. *Ferric hydroxide adsorption experiments. Increases in acidity due to enterokinase formed after 20 hours at 30°.*

cc. 0·1 *N* NaOH.

	Mother-liquor	Elution
Exp. a	0·00	0·36
Exp. b	0·06	0·50
Exp. c	0·08	0·48
Exp. d	0·07	0·40

The results given in Table II are of interest when it is remembered that the dipeptidase is also adsorbed by ferric hydroxide under these conditions and is therefore present together with the precursor in the elution. From the detailed scheme for the separation of the pancreatic enzymes of the tryptic and ereptic type given recently by Waldschmidt-Leitz [1931] it will be seen in fact that dipeptidase is the only known enzyme of this type which is present in the elution from the ferric hydroxide adsorptions. This raises the question of whether the transformation of precursor to enterokinase is itself dependent on

the dipeptidase. Waldschmidt-Leitz and Harteneck [1925] inferred the transformation to be an enzymic process, and Haldane and Stern's "Allgemeine Chemie der Enzyme" [1932] contains the suggestion that traces of "trypsin" may be responsible, but it is pointed out that this view is speculative. Although the balance of evidence in the present investigation appears to be more in favour of dipeptidase than of "trypsin," it is insufficient to settle the question decisively.

The effect of glycine.

Experiments were also carried out to examine the effect of added glycine upon the transformation process, since the action of dipeptidase upon glycylglycine is inhibited by this amino-acid [Euler and Josephson, 1926]. As Table III shows, glycine has apparently no measurable effect upon the formation of enterokinase.

Table III. *Increases in acidity due to formation of enterokinase after 18 hours at 30°.*

cc. 0·1 *N* NaOH.

	Without glycine	With glycine
Exp. a	0·78	0·74
Exp. b	0·72	0·70

The experiments were carried out with neutralised elution from the alumina, buffered with phosphate buffer to p_H 6·9. To a sample of 10 cc. of the buffered mixture was added 1 cc. of *N* glycine solution, and to a second sample of 10 cc. was added 1 cc. of distilled water. The formation of enterokinase in each of these samples after 18 hours at 30° was measured in the usual way.

If dipeptidase is assumed to be the responsible agent, the most that may be inferred from the above result is that glycine is not a product of the reaction. It is therefore of little value in helping either to confirm or disprove the suggestion.

Discussion.

Discussion of the mechanism of the change precursor → enterokinase is made especially difficult because at the present time the chemical nature of both precursor and enterokinase is unknown.

We do know, however, that enterokinase is thermolabile and has a critical increment for heat-inactivation of the same order as is observed with enzymes [Pace, 1931]. From this fact it seems reasonable to conclude that enterokinase possesses some form of colloidal support or carrier, which presumably therefore is also present in the precursor. If this supposition is correct it may be presumed that the transformation process itself is essentially some change occurring in the surface configuration of the carrier, for the magnitude of the critical increment of the process, 9500 calories, indicates that no very profound disruption is taking place. If an enzyme is responsible, it thus appears that the process probably involves the opening-up of relatively simple

surface linkages. But the experimental facts are insufficient to indicate the nature of the linkages involved, or the enzyme responsible. For although it has been shown that the process occurs in a solution containing dipeptidase alone (of the known pancreatic enzymes of the tryptic and ereptic type) the evidence available does not permit us definitely to attribute the change to this enzyme. Further experiments devised to test whether dipeptidase is responsible seem, however, to be a very suitable method of approach in any further investigation of this problem.

These results have been presented at a rather inconclusive stage of the investigation because circumstances prevent the writer from continuing work in this field.

Summary.

1. The critical increment of the process precursor → enterokinase is found to be 9500 cals per mole.

2. The precursor is shown to be adsorbable by ferric hydroxide, as dipeptidase is known to be. The elution following a ferric hydroxide adsorption contains therefore precursor and dipeptidase together. Enterokinase is ultimately formed in this elution.

3. The presence of glycine does not affect the rate of formation of enterokinase.

4. It is tentatively suggested that of the known pancreatic enzymes of the tryptic and ereptic type dipeptidase appears to be the one to which the formation of enterokinase might conceivably be ascribed. The low value of the critical increment suggests that the colloidal carrier is not involved, the change being restricted to certain surface groupings.

The writer is indebted to the Department of Scientific and Industrial Research for a grant to him as Research Assistant to Prof. W. C. M. Lewis, whose interest and advice he gratefully acknowledges.

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CLXXXVII. THE DETERMINATION OF BLOOD-PROTEINS BY ACID-ACETONE.

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During the course of experimental work with various methods for the determination of total protein in blood-plasma and serum, an endeavour was made to weigh the protein resulting from precipitation with trichloroacetic acid followed by washing with alcohol and ether. The results, compared with the direct gravimetric method of Knipping and Kowitz [1924] as modified by Starlinger and Hartl [1925], and the total nitrogen as determined by the Kjeldahl process, were invariably low, the following being a typical example.

	Total protein-nitrogen %
Kjeldahl direct, less N.P.N.	1·075
Precipitation with 95 % alcohol followed by Kjeldahl	1·11
Precipitation with 95 % alcohol; washed with ether; Kjeldahl	1·07
Gravimetric. Starlinger and Hartl. Factor 6·45	1·11
Precipitation with trichloroacetic acid and washing with alcohol and ether	0·66

On investigating the source of the loss it was found to be due entirely to the alcohol used for washing the precipitated protein.

Later it was found that 75–85 % acetone could be substituted for the alcohol and the conditions were worked out for obtaining the maximum yield of the acetone-soluble portion. The method ultimately adopted was as follows. 1 cc. of plasma or serum was diluted to 8·5 cc. in a 15 cc. centrifuge-tube and the total protein precipitated by the addition of 1·5 cc. of 20 % trichloroacetic acid drop by drop with constant shaking followed by inversion of the tube closed by the thumb. This is necessary to obtain a finely divided precipitate. The tube was centrifuged at a high speed for 10 min. and the supernatant liquid poured off; 0·25–0·30 cc. of 20 % $CCl_3.COOH$ was added; the volume made up to 2 cc. with water and then to 10 cc. with acetone. After stirring with a glass rod the precipitate and solvent were violently shaken and then centrifuged for 10 min. This usually resulted in a residue of insoluble protein occupying about 1 cc. from which the supernatant liquid could be poured off without loss of the deposit; the solvent was poured into a Kjeldahl flask and the precipitate again extracted with 80 % acetone containing 2·5 cc. of 20 % $CCl_3.COOH$ per 100 cc. The combined acetone washings were evaporated to dryness in a beaker of hot water and the residue in the tube used for the determination of total insoluble protein by the Folin-Kjeldahl technique.

The supernatant liquid from the first precipitation with trichloroacetic acid can, if desired, be used for the estimation of the N.P.N.

Whilst this work was in progress the proteins from egg-white were prepared by precipitation with ammonium sulphate followed by dialysis, but it was found that when these were examined by the above process, afterwards referred to as the S.P. (standard process) the globulin fractions were totally insoluble and that only a small fraction of the albumin was soluble.

These results opposed the idea, tentatively formed, that the acetone was merely separating the albumin and globulin, but when a number of routine hospital samples taken for the determination of the suspension stability had been examined, it was found that the acetone-soluble fraction showed some degree of correlation with the suspension stability and with the tryptophan content, both of which are related to the albumin/globulin ratio.

When pure albumin and globulin were prepared from human plasma, the albumin was found to be entirely soluble and the globulin totally insoluble in 80 % acetone containing trichloroacetic acid. Sheep- and ox-albumin were also found to be entirely soluble and in all subsequent work on pure proteins animal preparations were used.

The results so far obtained suggested that the process might be used as a clinical method for the determination of albumin and globulin, and various mixtures were accordingly prepared and analysed by the standard process with the following results.

Albumin (%)		
Theory	Found	Difference
6·4	5·4	−1·0
10·4	8·4	−2·0
16·0	13·6	−2·4
29·6	25·3	−4·3
46·6	43·3	−3·3
57·3	53·5	−3·8
79·6	77·4	−2·2

These results are fairly satisfactory but with very small amounts of globulin the precipitation of the globulin is somewhat delayed unless the concentration of the acetone is increased to 90 %. With 5 % of globulin (38 mg. of albumin and 2 mg. of globulin) and 80 % acetone the percentage error was 40–50 % but the separation of even 1 mg. of globulin from such a mixture cannot be regarded as entirely unsatisfactory.

A series of oxalated plasmata was analysed concurrently by the S.P. and by Howe's method [1921] in which sodium sulphate is used as the precipitant, with the following results, which are expressed as cc. of $N/10$ acid required to neutralise the ammonia formed from 1 cc. by the Kjeldahl process.

In the plasmata containing the higher amounts of albumin the differences were usually small and varied in direction but in the pathological samples containing an excess of globulin the Howe process invariably gave higher results.

Globulin	Albumin: Acetone-soluble	Albumin: Howe	Difference H. – A.
1·50	5·10	5·15	+0·05
2·00	4·85	4·50	−0·35
3·20	4·55	4·80	+0·25
2·85	4·40	4·25	−0·15
2·75	4·40	4·17	−0·13
3·40	4·15	4·40	+0·25
3·70	4·00	4·35	+0·35
3·25	3·97	4·03	+0·06
3·90	3·95	4·50	+0·55
2·80	3·90	4·25	+0·35
3·75	3·50	3·85	+0·35
4·10	3·35	3·55	+0·20
4·65	3·00	4·05	+1·05
5·40	2·75	3·13	+0·38

According to Merklen, LeBreton and Adnot [1931] complex lipoids hinder the precipitation of globulin from serum either by CO_2 or by neutral salts. On removing the lipoids by extraction with 80 % acetone, followed by dissolution of the precipitate in water and reprecipitation with acetone, they obtained the following results.

	Precipitant	Globulin. g. per litre: Ordinary serum	Globulin. g. per litre: Serum after acetone treatment
Pig blood	$MgSO_4(NH_4)_2SO_4$	44·7	52·7
		41·6	48·0
Chicken blood	$MgSO_4(NH_4)_2SO_4$	28·8	32·1
		30·4	38·8

Two points are worthy of note in connection with these results (1) the increase due to acetone extraction and (2) the variable results given by magnesium and ammonium sulphates both before and after acetone extraction. The latter point is also a feature of Howe's results and neither $MgSO_4$ nor $(NH_4)_2SO_4$ gave figures in accordance with the 21·3 % Na_2SO_4 recommended by him. Howe [1921, p. 101] records the following figures.

Precipitant	Albumin in filtrate. g. N per 100 cc.: Pig	Goat	Horse
Na_2SO_4 21·3 %	0·393	0·399	0·443
$(NH_4)_2SO_4$ half saturated	0·526	0·443	0·477
$MgSO_4$ saturated	—	0·323	0·385

The percentage differences, calculated on the Na_2SO_4 results are

$(NH_4)_2SO_4$	+34	+11	+ 8
$MgSO_4$	—	−19	−13

In a second paper, Howe [1921, p. 113] gives further figures for these types of blood which show variations of similar magnitude and direction, $(NH_4)_2SO_4$ always giving larger, and $MgSO_4$ smaller, amounts of albumin than Na_2SO_4.

It is probably generally appreciated that there are, in the usual precipitation methods, small inherent errors such as the dilution factor and the volume contraction (1–2 % in the case of adding half saturated $(NH_4)_2SO_4$ to an equal volume of serum) but the variations in the above figures are of a

very different order and strongly suggest that when applied indiscriminately to normal and pathological serum and plasma, such methods are somewhat empirical.

The effect of acetone extraction carried out according to the method of Merklen *et al.* [1931] was determined on 3 samples of human plasma, using Howe's process and the acetone-acid method previously described. The results, expressed as nitrogen in terms of $N/10$ acid, were:

Sample	T.P.N.	Method	Albumin-nitrogen	
			Before acetone treatment	After acetone treatment
I	7·33	Na_2SO_4	4·20	3·62
		Acetone, S.P.	3·90	4·00
II	7·78	Na_2SO_4	4·20	3·46
		Acetone, S.P.	3·75	4·02
III	6·42	Na_2SO_4	2·43	1·40
		Acetone, S.P.	1·20	0·95

The acetone treatment was carried out as recommended by Merklen, *viz.* 1 cc. of plasma was diluted with 1 cc. of water, 8 cc. of acetone added and centrifuged. The precipitate was dissolved in 2 cc. of water and made up to 10 cc. with acetone. After centrifuging it was deemed desirable to remove the acetone mixed with the precipitate as this might affect the subsequent precipitation with Na_2SO_4. This was effected in a vacuum desiccator, care being taken not to remove all the water. The precipitate was then dissolved in water at 37°; the volume made up to about 11 cc.; 3·05 g. of anhydrous Na_2SO_4 added; and the volume made up to 15 cc. after the sulphate had completely dissolved. After incubation at 37° for 4 hours the precipitate was filtered off and the N determined in the filtrate.

It might be suggested that the acetone treatment would produce some irreversible coagulation of the albumin, but this is not supported by the results obtained by the standard process nor by the fact that a mixture of pure albumin and globulin gave practically identical results with Na_2SO_4 before and after treatment with acetone.

The above results definitely indicate that acetone removes some substance which materially interferes with the precipitation of globulin by Na_2SO_4 and that this substance is present in greater amount in plasma showing a low albumin/globulin ratio. This substance also affects the results obtained with the acid-acetone process but to a smaller degree.

Attempts to produce this effect synthetically have so far proved unsuccessful. The acetone treatment would more or less completely remove the lipoids together with some lecithin but it was found that the addition of a lecithin emulsion to a mixture of pure albumin and globulin had no effect; the same results were obtained by the Na_2SO_4 and acid-acetone processes both before and after acetone treatment.

A further attempt was made by adding the substances extracted by 80 % acetone. 100 cc. of ox-serum were precipitated by adding 400 cc. of acetone,

the proteins filtered off, and the acetone removed from the filtrate at room temperature. The resulting emulsion had also no effect on the precipitation of globulin from a mixture of this substance with pure albumin.

Albert Fischer [1932] has recently stated that on the addition of anti-prothrombin (heparin, Howell) to albumin it forms a complex which is identical with genuine globulins when considered in relation to solubility, precipitation with weak acids, denaturation, salting out, solubility in excess of heparin, and isoelectric point.

The claims regarding the effect on denaturation and salting out with Na_2SO_4 could not be confirmed in the author's laboratory with the only heparin preparation available (Parke Davis and Co.).

These failures to affect the precipitability of globulin are merely negative evidence and do not disprove the hypothesis that the increased amount of globulin, as determined by the usual salting out methods after acetone treatment, is due to the removal of lipoids and allied substances. These substances are not present in plasma and serum in the free condition, nor is it probable that the various proteins known as euglobulin, pseudoglobulin and albumin exist in serum as such; they are probably present as one or more large complexes which break down in a manner directed by the relatively small amount of substances associated with the proteins. As to the nature of this process practically nothing is known, though the recent work of Schmitz and Wulkow [1932], who employed spectroscopic methods, suggests that in certain pathological sera not only is the micellar system changed but the structure of the individual components is also altered.

If the adsorption of phosphorus-containing substances is capable of altering materially the solubility of globulin, as is indicated by the work of Chick [1914], it is quite possible that there may also be substances which render the whole group of globulins less amenable to the precipitating action of salts.

There is also some collateral evidence that the usual methods for the determination of albumin and globulin in serum and plasma are not satisfactory. Lang [1930], on estimating the serum-protein-sulphur, found that the results differed from those calculated from the albumin and globulin content: in 100 cases, 46 agreed with the theoretical within 10 % but in the remaining 54, variations up to 50 % were obtained. In the latter either the sulphur content of the individual fractions varied or the estimations of the albumin and globulin were inaccurate. Probably both factors were involved, for whilst the above results throw suspicion on the latter one, some unpublished work of the author on the tryptophan content of pathological plasma suggests that one or more of the proteins has changed "qualitatively."

The above data indicate that whilst the usual salting out methods for the estimation of albumin and globulin may yield accurate results with mixtures of pure proteins, they are unreliable when applied to pathological sera and plasmata and that the results vary with the nature of the salt employed.

The suggested acid-acetone process, while giving slightly low results for albumin in mixtures of pure proteins, is probably just as accurate as salt methods with normal sera and plasmata and gives much smaller errors with pathological samples.

Various factors affecting the standard process.

(1) *Effect of salts.* This was determined by adding varying amounts of salts to 1 cc. of a well dialysed albumin solution and determining, by the standard process, the amount rendered insoluble.

Added salt	% of albumin insoluble
1 cc. 0·9 % NaCl	10
Ringer's solution, 6 cc.	42
,, ,, 3 cc.	32
,, ,, 1 cc.	1
$CaCl_2$ (7 mg. per 100 cc.), 1 cc.	Nil
Na_2SO_4, 100 mg.	10
,, 85 ,,	9
,, 65 ,,	2
,, 50 ,,	1
,, 25 ,,	0·5
,, 10 ,,	0·5

Although the addition of 1 cc. of isotonic NaCl rendered a very appreciable amount of the albumin insoluble in 80 % acid-acetone, the results obtained with Ringer's solution suggested that the salts normally present in plasma and serum would not interfere with the separation of albumin and globulin in such solutions. This was confirmed by the analysis of four samples of plasma before and after dialysis and also by removal of the salts by ultra-filtration.

(2) *Effect of added acid.* This is shown by the following results obtained with oxalated human plasma.

Added acid cc. 20 % $CCl_3.COOH$	p_H centrifuged solution	cc. $N/10$ acid equal to nitrogen as	
		Globulin	Albumin
Nil	2·0	1·45	—
0·08	1·8	1·95	—
0·17	1·6	2·15	—
0·25	1·5	2·10	4·7
0·30	1·45	2·10	—
Howe's process	—	2·35	4·45

The p_H in this, and in all subsequent work, was estimated colorimetrically and no allowance was made for the protein error. This, however, is very small as the maximum concentration was under 0·4 %.

When the added acid was less than 0·17 cc. (p_H 1·6) the globulin was incompletely removed from solution and the acetone was slightly turbid after centrifuging for 10 mins. 0·25–0·30 cc. always gave bright clear solutions and was the amount adopted in all later work.

(3) *Other acids.* The trichloroacetic acid used in this process is not specific since dichloroacetic acid was found to give equally good results; sulphosalicylic acid, another protein precipitant, gave very poor results.

The comparative results on 1 cc. of human plasma were:

	Protein-N as cc. $N/10$ acid		
	Trichloroacetic	Dichloroacetic	Sulphosalicylic
Acetone-soluble	3·25	3·40	1·40
Acetone-insoluble	4·55	4·45	6·10
	7·80	7·85	7·50
N.P.N.	0·25	0·25	0·55

It was found that whilst 1·5 cc. of 20 % $CCl_3.COOH$ was quite sufficient for the complete precipitation of the protein, about 3 cc. of $CHCl_2.COOH$ were required. Sulphosalicylic acid did not precipitate the protein completely and also failed to bring all the albumin into solution. With a solution of pure albumin sulphosalicylic acid acted similarly, only about 60 % of the original albumin being found in the acetone solution.

In the later stage, dichloroacetic acid, but not the more weakly dissociated acetic acid, may be substituted for trichloroacetic acid. It was found that when 1 cc. of a 4 % solution of albumin was mixed with an equal volume of water and 8 cc. of acetone added, complete solution of the precipitate could not be obtained by the addition of acetic acid. When the acetone concentration was reduced the precipitate dissolved completely at a higher p_H.

On the addition of a more strongly dissociated acid, HCl, to a solution of albumin and making the acetone content up to 80 % by volume, the protein remained in solution until the p_H was reduced below 1·5 and was completely precipitated at about p_H 0·7 (*vide* Fig. 1). Przyłęcki [1931], in experiments on the heat-denaturation of serum-albumin in the presence of HCl followed by the addition of alcohol (85 % in final solution), found coagulation at p_H 4·5, opalescence at p_H 4·2–3·9, clear solutions at p_H 3·2–1·4, turbidity when the p_H was <1, and acid coagulation at 0·5–0·7 *M*.

When trichloroacetic acid was used with 80 % acetone, serum-albumin gave perfectly clear solutions even at p_H 1·25 and 0·8 and these remained clear until 14 days later when they became faintly opalescent; after 16 days a gelatinous precipitate formed and slowly settled down.

Solutions of serum-albumin when treated by the standard process also gave at p_H 1·6–1·05 clear solutions which were stable for days.

The results indicate that HCl may only be substituted for $CCl_3.COOH$ in the second stage of the process if the p_H is rigidly controlled within narrow limits. Below p_H 1·5 the albumin becomes insoluble and above p_H 1·7 globulin becomes soluble. This is well illustrated by the following figures obtained with human plasma.

Added acid	p_H	% of total protein insoluble in acetone
$CCl_3.COOH$. 20 % 0·3 cc.	1·5–1·6	63
HCl *N* 0·37 cc.	1·2–1·3	97
HCl *N* 0·25 cc.	1·5	77
HCl *N* 0·12 cc.	1·6	69
HCl *N* 0·07 cc.	1·7	61
Nil	1·8	55

Paraglobulin (pseudoglobulin) when treated with HCl and 80 % acetone, *i.e.* without preliminary precipitation with $CCl_3.COOH$ (afterwards referred to as Process II) is completely precipitated (*vide* Fig. 2). With this concentration of acetone the usual isoelectric point is completely obliterated and clear solutions can only be obtained by the addition of alkali. On reducing the acetone concentration the isoelectric zone appears first as a broad band which narrows to a point on further reduction of the acetone concentration.

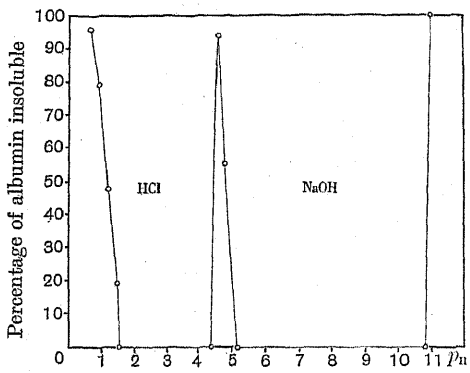


Fig. 1. Albumin and 80 % acetone.

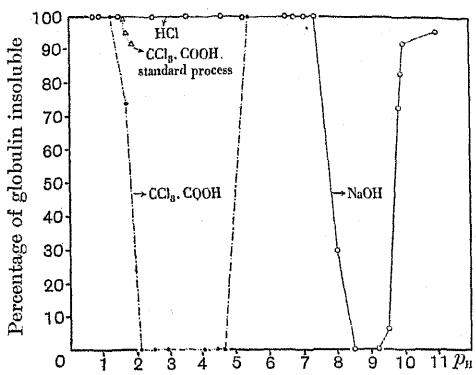


Fig. 2. Pseudoglobulin and 80 % acetone. Process II.

When $CCl_3.COOH$ is used in Process II with paraglobulin, precipitation commences immediately at about p_H 2·0–2·1 and is complete at about p_H 1·2.

When treated by the standard process, paraglobulin is almost entirely (about 90 %) irreversibly coagulated by the preliminary precipitation and on adding sufficient $CCl_3.COOH$ to bring the p_H to 1·6–1·5, the precipitate becomes quite insoluble in 80 % acetone.

The essential facts emerging from these experiments so far as they relate to the standard process are that after precipitation with $CCl_3.COOH$, serum-albumin is completely soluble in 80 % acetone even when the p_H is reduced to <1 by adding $CCl_3.COOH$ and that serum-paraglobulin becomes entirely insoluble when the p_H is reduced to 1·6–1·5. These results obtained with pure proteins accord with those obtained with plasma and recorded above. The amount of $CCl_3.COOH$ required to ensure the correct p_H is 0·25–0·30 cc. of a 20 % solution per cc. of plasma.

The agreement between the results obtained with pure albumin and paraglobulin on the one hand and with plasma on the other suggest that fibrinogen and euglobulin are also completely precipitated under the conditions obtaining in the standard process.

(4) *Acetone concentration.* At an early stage in these experiments the effect of varying the acetone concentration in the standard process was determined on solutions of serum-albumin and paraglobulin and the results are shown in Figs. 3–6.

In these diagrams the curves marked immediate were drawn from the results obtained by centrifuging the solutions immediately after the acetone had been added and thoroughly mixed with the precipitated protein. The other curves represent the results similarly obtained after the time interval stated.

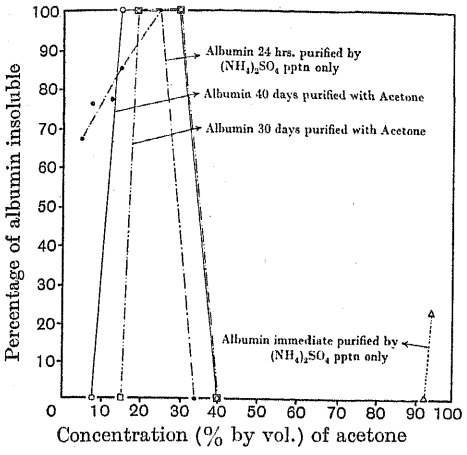


Fig. 3. Albumin and acetone standard process.

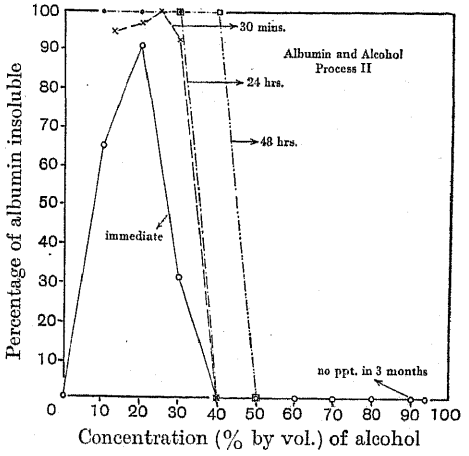


Fig. 4.

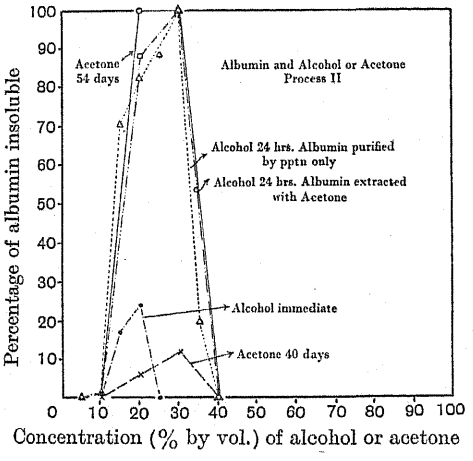


Fig. 5.

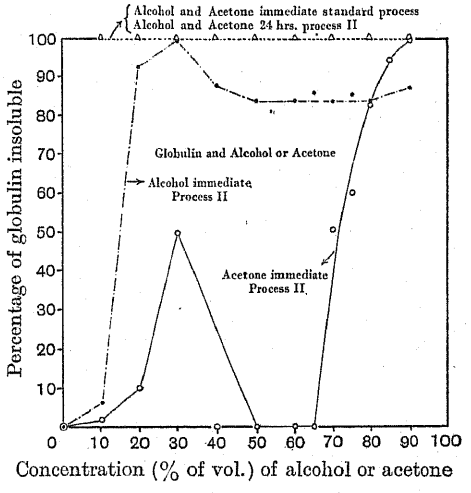


Fig. 6.

It will be seen (Fig. 6) that whilst globulin is entirely insoluble in all acetone concentrations from 10 to 90 %, the albumin curves (Fig. 3) show a distinctly anomalous feature at the lower concentrations. Between 25 and 30 % acetone the incompletely purified albumin became quite insoluble but only a further 10 % was necessary to produce complete solution.

On the advice of Sir F. G. Hopkins, these experiments were repeated with

the substitution of alcohol for acetone and the results are given in Figs. 4–6, together with similar curves obtained with Process II, *i.e.* without the preliminary precipitation with $CCl_3.COOH$.

It will be seen that all the curves, with the exception of those showing total insolubility at all concentrations, exhibit the same node at about 25–30 % whether the solvent be alcohol or acetone, and the solute albumin or globulin.

The curves suggested a change in the physical properties of the solvent at about 30 % concentration by volume and as surface tension is a factor in precipitation a search was made for relevant data.

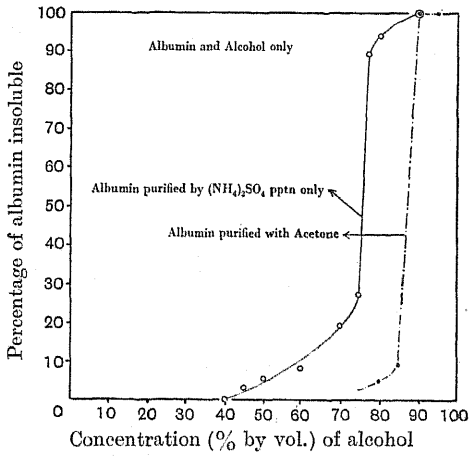


Fig. 7.

Morgan and Scarlett [1917] determined the surface tension of acetone-water mixtures by the drop weight method and suggested that the results indicated the formation of a definite compound $(CH_3)_2.CO, 10H_2O$. This was based on the theory of Denison [1912] which, as stated by Morgan and Scarlett, is that "the maximum deviation of the property (surface tension) as observed from that calculated by the mixture law is invariably found to exist at a concentration of the mixture corresponding to a definite simple molecular proportion of the two constituents, and this same concentration continues to exhibit the maximum deviation at all temperatures." The case of acetone-water mixtures was studied intensively by Morgan and Scarlett and the maximum deviation was found at 24–25 % by weight (30 % by vol.) of acetone and was constant between 0° and 45°.

The surface tension of alcohol-water mixtures has been investigated by Grunmach [1912]. Calculations based on Grunmach's data give a maximum deviation from the mixture law at 26·5 % of alcohol by weight by the dynamic method and 31 % by weight by the static one. The maximum deviation obtained from the data in the Smithsonian tables, probably derived by a static method, is at 29·5 % by weight.

When the obvious difficulties connected with the protein precipitation method are taken into consideration, the agreement with the surface tension methods is very satisfactory and leaves little doubt as to the cause of the nodes in the various curves.

In the construction of Figs. 3 and 5, two different preparations of albumin were used, one purified by repeated precipitation from half saturated $(NH_4)_2SO_4$ followed by prolonged dialysis, and the other prepared in a similar manner but precipitated with cold acetone prior to dialysis. With alcohol as the precipitant (Fig. 5) the two albumins gave very similar results, but with acetone (Figs. 3 and 5) the gelation and precipitation of the purer specimen were very markedly delayed.

The acetone-treated albumin is also less susceptible to precipitation by alcohol in the absence of acids and alkalis (*vide* Fig. 7) and a well purified specimen of serum-albumin requires a high concentration of alcohol for complete precipitation.

In accordance with the experience of Mayer and Terroine [1907] no specimen was obtained which could not be precipitated with alcohol, but it was found that if traces of acid were present even a concentration of 95 % of alcohol produced no precipitate within 5 months.

Various proteins.

An examination of various proteins by the standard acetone process has yielded the following results.

	Acetone-soluble (%)	
	Albumin	Globulin
Human serum	100	Nil
Ox serum	100	,,
Sheep serum	100	,,
Horse serum	100	,,
Egg	Nil	,,
Caseinogen	29–30	
Globin	65	
Haemolysed erythrocytes	43–69	

With proteins from mammalian blood the acetone value coincides with the solubility in half saturated $(NH_4)_2SO_4$, but egg-albumin behaves anomalously, the acetone solubility being that of a globulin. The reason for this will be discussed later.

Some of the earlier preparations of egg-albumin showed a slight but definite (up to 10 %) solubility by the standard process, but it was later found that by carefully avoiding an excess of acetic acid during the precipitation by the method of Hopkins and Pincus, followed by recrystallisation and a short rapid dialysis through a fairly permeable collodion membrane, the acetone-soluble content could be reduced to less than 1 %.

By adding further acetic acid to the filtrate from the egg-albumin, an acid albumin with 4 % solubility in acid-acetone was obtained and by adding

further ammonium sulphate to the filtrate from this protein a preparation was obtained with an acetone value of 10 %.

These higher values may be due either to the presence of acid albumins with appreciable acetone values or to partial hydrolysis of the egg-albumin during its longer contact with a greater concentration of acid.

Basis of standard process.

The process for the separation of blood-albumin and globulin depends primarily upon the difference in the velocity of the denaturation process and the coagulation of the denatured protein.

When albumin is precipitated with $CCl_3.COOH$ under the conditions obtaining in the S.P., the precipitate is entirely soluble in water and does not give a nitroprusside reaction after reduction with cyanide. Serum-globulin, when similarly treated, gives a precipitate which is about 90 % insoluble in water and contains the —S—S— group.

These results indicate that whilst globulin is denatured and irreversibly coagulated neither process occurs with albumin within the period of time required to complete the determination.

A slow denaturation of serum-albumin does occur, however, and in one experiment with ox-albumin gelation took place after 54 days and the protein separated out as a precipitate 7 days later. This precipitate gave a marked nitroprusside reaction after reduction with cyanide.

Egg-albumin.

The anomalous behaviour of this albumin when treated by the standard process is apparently due to the rapidity with which it undergoes denaturation and irreversible coagulation during the preliminary treatment with $CCl_3.COOH$. The precipitate so obtained was insoluble in water and gave a marked sulphydryl reaction.

The complete change of ovalbumin to the coagulated state (denaturation; lyophile-lyophobe) under the influence of urea and urethane in the vicinity of the isoelectric point was found by Hopkins [1931] to proceed much more rapidly than with blood-proteins. With high concentrations of urea he obtained an intense —S—S— reaction at once with blood-proteins and suggested that although the chemical changes associated with denaturation proceed at once with blood-proteins the colloids change more slowly than ovalbumin from the lyophile to the lyophobe condition.

The difference in the nitroprusside reactions with blood-proteins may be due to the marked difference in the p_H in the experiments, that used by the author being much more acid than was employed by Hopkins and sufficient to convert the proteins into the acid compounds.

If the acid albumin precipitate obtained with $CCl_3.COOH$ were dissolved in 80 % alcohol and neutralised, the protein slowly precipitated and gave a definite —S—S— reaction.

It is evident that the complete change to the lyophobe state takes place with greater velocity with both electrically neutral and acid ovalbumin than with serum-albumin.

When treated as in Process II, ovalbumin gave very different results with $CCl_3.COOH$; on diluting 0·5 cc. of a 3 % ovalbumin solution to 2 cc. and adding 8 cc. of acetone followed by 0·25 cc. of 20 % $CCl_3.COOH$, no precipitate was obtained and the liquid remained clear until 48 hours had elapsed. On adding the same amount of $CCl_3.COOH$ before the addition of acetone, 39 % of the ovalbumin was precipitated at once.

On adding HCl to the ovalbumin and then acetone, the following results were obtained:

Added acid		p_H	% albumin insoluble
HCl	0·3 cc. *N*	1·25	63
,,	0·2 cc. *N*	1·6	11
,,	0·1 cc. *N*	1·85	Nil
$CCl_3.COOH$	0·1 cc. *N*	2·1	4

With 0·5 cc. of 3 % ovalbumin and 0·1 cc. of *N* HCl and varying amounts of acetone the results were:

Acetone concentration (%)	% albumin insoluble
80	Nil
85	58
90	100
95	100

Lepeschkin [1922] found that if various acids were tested at the same molar concentration, the denaturation velocity was determined by the H ion concentration and that the nature of the acid had no influence, but the above results with 0·1 *N* HCl and $CCl_3.COOH$ indicate that this generalisation does not hold completely and that some additional factor is involved in the case of such an efficient protein precipitant as trichloroacetic acid.

Summary.

1. A process is suggested for the estimation of serum-albumin and globulin by precipitation with trichloroacetic acid and extraction of the precipitate with acidified acetone.

2. Treated by this process, serum-albumin is entirely soluble and serum-globulin is entirely insoluble.

3. The process gives comparable results with those obtained by Howe's method (Na_2SO_4 precipitation) for normal sera and plasmata, but gives lower figures for globulin in pathological specimens.

4. Experiments with acetone extraction indicate that Howe's process gives low globulin results with pathological specimens and that more accurate ones are obtained by the acid-acetone method.

5. In experiments with pure proteins and varying concentrations of acetone (or alcohol) a node was found in the curves at 25–30 % of acetone

(or alcohol) corresponding to the concentration at which the surface tension shows the maximum deviation from the mixture law.

6. All the blood-albumins tested (human, sheep, ox and horse) were entirely soluble in acid-acetone, but egg-albumin was insoluble.

7. The solubility of the various proteins is determined by the rate of denaturation and coagulation in acid-acetone.

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CLXXXVIII. THE QUANTITATIVE DETERMINATION OF VITAMIN D BY MEANS OF ITS GROWTH-PROMOTING PROPERTY.

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A certain amount of distrust has sometimes been felt for those methods of estimation of vitamin D in which the basal diet of the rats either had an abnormally high calcium, low phosphorus content (*e.g.* the McCollum diet 3143 and the Steenbock diet 2965, in both of which the ratio Ca:P is about 4:1), or was devoid of various essentials for normal nutrition (*e.g.* the Pappenheimer diet on which rats do not grow). Dr J. H. Burn suggested that it should be possible, with an adequate basal diet, to use the growth-promoting property of vitamin D [Steenbock, Nelson and Black, 1924] as a means of estimating that vitamin with a degree of accuracy equal to that obtained in the estimation of vitamin A by means of its growth-promoting property [Coward, Key, Dyer and Morgan, 1930; 1931; Coward, 1932]. We therefore decided to use the same basal diet as that used in our vitamin A determinations but, instead of giving a supplement of vitamin D throughout the preparatory and test periods and graded doses of vitamin A during the test period, we gave a supplement of carotene (for vitamin A) throughout the preparatory and test periods and graded doses of vitamin D during the test period. We found that the rats ceased to grow on the diet rich in carotene but free from vitamin D in varying lengths of time, and resumed growth on being given vitamin D. The response was graded to the dose of vitamin D given. We thus had a method of estimation of vitamin D in which the basal diet of the animals supplied all needs except that for vitamin D. It is thus free from the objections sometimes made to the diets in general use for vitamin D determinations. While these objections still seem to be theoretically sound, they do not appear to be serious from the practical considerations of vitamin D estimations, for we have obtained the same results in estimating the vitamin D content of substances by using the line test and the growth test.

Method.

The rats used in these experiments were pure-bred albinos of about 30 g. weight, from our own colony. The members of each litter were kept together

in one cage, bedded on sawdust and given tap water and the basal diet *ad lib.* The basal diet consisted of:

Caseinogen, light white (B.D.H.)	15 %
Dextrinised rice starch	73 %
Dried yeast	8 %
Salt mixture (Steenbock's 40)	4 %

Each rat was given a dose of carotene in olive oil three times a week throughout the whole experiment. During the preparatory period (*i.e.* the time taken for the rats to exhaust their reserves of vitamin D) they were weighed twice a week. As each one became steady in weight it was placed in a separate cage for the test period of 5 weeks. Weighings were then made once a week.

Two experiments have been carried out. In the first the carotene was given as 0·02 mg. (and later as 0·04 mg.) daily of the light petroleum-soluble fraction of dried carrots. It was dissolved in olive oil so that 0·02 g. of the solution (the amount of one drop delivered from our dropping tubes for doses of cod-liver oil) contained the required dose. In the second, crystalline carotene (0·04 mg. in 0·02 g. olive oil) prepared by ourselves, or later supplied by the British Drug Houses, Ltd., was used. This was not easily soluble in olive oil but the insoluble part made a suspension satisfactory enough for the purpose of this experiment.

Results.

(*a*) *Behaviour of the rats on the basal diet alone.*

Some of our rats grew very little after being given this diet. They did not, however, decline in weight and it was found to be quite safe to keep them on the basal diet for at least a month before giving them doses of vitamin D. Other rats increased 20 to 30 g. or even as much as 60 to 70 g. before becoming steady in weight and several had to be discarded at about the weight of 110 g. as too heavy for use in the test. It was evident that the reserves of vitamin D in the young rat varied in different individuals and in different litters. Moreover, none of the rats grew as rapidly as those in the preparatory periods of a vitamin A test, nor did they decline rapidly and die when their reserves were exhausted. Certain individuals which were given no dose of vitamin D remained practically level in weight for many weeks, and died without any great loss in weight. Occasionally a rat would resume growth without any apparent cause. In fact the behaviour of these rats recalled vividly the behaviour of rats in those early vitamin A tests which were made before vitamin D was recognised as a growth-promoting substance and added to the basal diet used in vitamin A tests. In those rats, almost certainly cessation of growth had been due to a lack of vitamin D rather than to a lack of vitamin A and growth had been resumed only when the test material contained vitamin D.

(b) Response to doses of vitamin D.

Occasionally the response to the daily dose of vitamin D was unnoticeable during the first week, but generally it was immediate and continued smoothly throughout the test period. This was prolonged for 8 weeks in the early part of the work, but later was cut down to 5 weeks. Doses of 0·01, 0·05, 0·1, 0·2, 0·25, 0·4, 0·75 and 0·8 unit of vitamin D daily were tested, each dose being contained in 0·02 g. olive oil. These have given responses graded logarithmically to the dose. That is to say, the mean increases in weight of the groups of rats plotted against the logarithms of the doses of vitamin D given fall practically on a straight line. The calculation of the straight line most accurately fitting these observations gives the equation of the curve of response between mean increase and dose [Fisher, 1930].

The curves are:

$$y = 92{\cdot}82 + 30{\cdot}02 \log x \text{ (bucks)},$$
$$y = 63{\cdot}14 + 22{\cdot}74 \log x \text{ (does)},$$

where y = the mean increase in weight in g. of the groups of rats,

and x = the dose of vitamin D in units.

In this experiment carotene in a daily dose of 0·04 mg. in 0·02 g. olive oil was supplied during the whole of the preparatory and test periods.

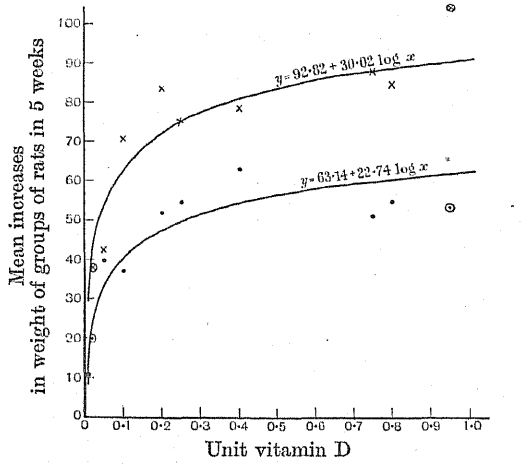


Fig. 1. Curves of response to doses of vitamin D of rats which had ceased to grow on a diet containing all factors known to be essential to growth (including a complete salt mixture) but lacking in vitamin D. The curves were determined by calculating the best straight line through the points relating mean increases in weight to the logarithms of doses of vitamin D given:

× = Mean increases in weight of groups of bucks in response to doses of the international standard preparation of vitamin D.

• = Mean increases in weight of groups of does in response to doses of the international standard preparation of vitamin D.

⊗ and ⊙ = Mean increases of groups of bucks and does respectively in response to doses of a commercial sample of irradiated ergosterol already assayed by the line test.

Tests of the curves.

A commercial sample of irradiated ergosterol in maize oil was estimated in this laboratory (and also in two others) by means of the line test, to contain 2,000,000 units of vitamin D per g. Before the estimation was completed, however, we had begun to test a dose of 0·0005 mg. of the solution by means of the growth test. When we realised the exceedingly high potency of the preparation, we began to test a lower dose, 0·00001 mg. of the solution. The results are summarised in Table I B and the mean increases in weight have

Table I.

	Dose of vitamin D units	No. of ♂ rats surviving 5 weeks	No. of ♂ rats dead in 5 weeks	Mean increase in wt. in 5 weeks g.	$\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$	No. of ♀ rats surviving 5 weeks	No. of ♀ rats dead in 5 weeks	Mean increase in wt. in 5 weeks g.	$\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$	Average increase* g.
(*a*) 0·04 mg. carotene as source of vitamin A:										
A	0·01	0	0	—	—	2	1	11·0	1·41	11·0
	0·05	5	2	42·4	14·37	5	0	39·8	3·12	41·1
	0·1	5	2	70·8	7·01	6	0	37·5	12·98	54·15
	0·2	5	0	83·6	14·37	5	1	52·0	5·20	67·8
	0·25	1	1	75·0	—	2	0	54·5	4·95	64·75
	0·4	5	0	78·4	25·97	6	0	63·1	15·69	70·75
	0·75	1	1	88·0	—	1	0	51·0	—	69·5
	0·8	4	0	84·75	9·22	5	2	54·8	9·42	69·77
	10·0	2	0	84·0	36·77	1	0	57·0	—	70·5
B	0·00001 mg. I.E. (=0·02 unit)	1	0	38·0	—	6	0	20·2	3·19	29·1
	0·0005 mg. I.E. (=1·0 unit)	5	0	104·2	12·21	6	0	53·3	5·97	78·75
(*b*) 0·04 mg. light petroleum-soluble fraction of dried carrot as source of vitamin A:										
C	No dose	4	3	17·25	8·35	3	5	0	8·54	8·625
	0·01	6	1	3·1	9·87	2	0	5·5	4·95	4·3
	0·02	5	1	9·8	7·70	7	0	5·7	3·38	7·75
	0·05	6	0	10·7	4·86	4	1	14·5	7·23	12·6
	0·1	7	1	15·0	12·73	7	2	24·6	12·23	19·8
	0·5	7	1	41·4	21·42	2	1	40·5	0·71	40·95
	1·0	6	1	36·0	23·70	3	0	36·7	11·85	36·35

* This is the simple average of the values obtained from the bucks and does in each group. It seemed unsound to calculate the weighted means in the tests where large doses of vitamin D were given and the bucks made much greater increases in weight than the does.

Table II.

Dose of solution of I.E. mg.	No. of ♂ rats on test	Mean increase in wt. in 5 weeks g.	Dose of standard which would have brought about this increase, units	Dose of standard equiv. to 1 g. units	No. of ♀ rats on test	Mean increase in wt. in 5 weeks g.	Dose of standard which would have brought about this increase, units	Dose of standard equiv. to 1 g. units
0·00001	1	38	0·0149	1,490,000	6	20·2	0·0129	1,290,000
0·0005	5	104	2·3572	4,714,400	6	53·3	0·3692	738,400

The weighted mean of the values found from the bucks and does respectively on the two doses is 2,068,500 units per g., which is near to the estimation made by the line test.

been plotted on the curves of response of the bucks and does in Fig. 1, assuming, on the strength of the result obtained by the line test, that the lower dose contained 0·02 unit and the higher dose contained 1·0 unit.

The estimation of the potency of the solution would, however, be made direct from the curves of response by substituting the found values of y, and

taking the weighted mean of the calculated values for x. Thus the potency of this sample was found to be 2,068,500 units per g. by the growth method, which confirms the figure of 2,000,000 units found by the line test.

THE DEGREE OF ACCURACY OBTAINABLE BY THIS METHOD.

The standard deviation (σ) *of the test.*

The standard deviation $\left(\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}\right)$ of each result has been calculated (Table I). The mean (weighted) variances for the bucks and does respectively have also been calculated (Table III).

Table III.

Average variance $= \sigma^2 = \frac{\Sigma d^2}{N-m}$ (N = no. of observations, m = no. of tests)

	♂	♀
Tests in which vitamin A was supplied as the light petroleum-soluble fraction of dried carrots	223·06	74·93
Tests in which vitamin A was supplied as crystalline carotene (including two not quoted in this paper)	282·7	77·08
Weighted mean of whole series	249·5	76·31

Thus the standard deviation of the mean increase in weight during 5 weeks of bucks which have become steady in weight on a diet deficient only in vitamin D, and have then been given graded doses of that factor, is $\sqrt{249·5} = 15·80$. The standard deviation of does treated similarly is $\sqrt{76·31} = 8·74$. The value for the bucks is very similar to that found in the vitamin A (growth-promoting) test, which was 14·72. The value for the does is distinctly lower than the one found in the vitamin A test (11·02).

The standard error (ϵ) *of the mean.*

The standard error of the mean has been calculated from the formula, $\epsilon = \frac{\sigma}{\sqrt{n}}$ where σ = the standard deviation, n = the number of animals used in the test. When $n = 10$ (a reasonable number to use for a vitamin test) $\epsilon = 5·00$ for the bucks and 2·74 for the does. The degree of accuracy obtainable by the use of 10 animals has been calculated and stated in Table IV. The doses of vitamin D required to bring about mean increases in weight of 60 g. and 40 g. in groups of bucks and does respectively (seen from the curves in Fig. 1 to be convenient) have been calculated. The doses of vitamin D required to bring about mean increases of $60 \pm \epsilon$ and $60 \pm 2\epsilon$ in groups of 10 bucks and of $40 \pm \epsilon$ and $40 \pm 2\epsilon$ in groups of 10 does have been determined. The ranges of these doses have then been calculated as percentages of the original dose and the degree of accuracy of the test obtained. Thus, with 10 bucks, a result within 47 % above, and 32 % below the true value would be obtained 2 out of 3 times, and within 116 % above and 54 % below,

21 times out of 22. Similarly with 10 does a result within 32 % above and 24 % below the true value, would be obtained 2 out of 3 times and within 75 % above and 43 % below, 21 out of 22 times (see Table IV).

Table IV.

When the number of rats is 10, $\epsilon = 5{\cdot}00$ for bucks and $\epsilon = 2{\cdot}76$ for does.

Mean increase ♂ g.	Mean increase ♀ g.	Equiv. dose of vitamin D ♂ unit	Equiv. dose of vitamin D ♀ unit	Range of mean increase when $n=10$ ♂ g.	Range of mean increase when $n=10$ ♀ g.	Range of equiv. dose of vitamin D ♂ unit	Range of equiv. dose of vitamin D ♀ unit	Percentage range of equiv. doses ♂	Percentage range of equiv. doses ♀
				(*a*) $\pm\epsilon$					
60	40	0·0806	0·0960	65	42·76	0·1184	0·1270	146·9	132·3
				55	37·24	0·0550	0·0726	68·3	75·6
				(*b*) $\pm 2\epsilon$					
				70	45·52	0·1737	0·1679	215·5	174·9
				50	34·48	0·0375	0·0549	46·5	57·2

The logarithmic variance of the test.

The standard deviation of the logarithm of the result is obtained by dividing σ by the slope of the line connecting the response with the log (to the base 10) of the dose. The curves relating the response to the log of the dose are

$$y = 92{\cdot}82 + 30{\cdot}02 \log x \text{ (bucks)},$$
$$y = 63{\cdot}14 + 22{\cdot}74 \log x \text{ (does)}.$$

Then the standard deviation (λ) of the logarithm of the result is

$$\frac{15{\cdot}80}{30{\cdot}02} = 0{\cdot}526 \text{ for bucks} = \lambda_1,$$
$$\frac{8{\cdot}76}{22{\cdot}74} = 0{\cdot}385 \text{ for does} = \lambda_2.$$

The logarithmic variance of the test is then

$$0{\cdot}276676 \text{ for bucks } (\lambda_1^2),$$
$$0{\cdot}148225 \text{ for does } (\lambda_2^2).$$

The error of the test is $\frac{\lambda}{\sqrt{n}}$. As λ_1^2 is about twice λ_2^2, about twice as many bucks as does are required in this test for a given degree of accuracy.

Significant differences between the mean increases in weight of groups of rats may be calculated as in the vitamin A tests.

The relative degrees of accuracy obtainable by the growth method for vitamin D and for vitamin A are indicated in a comparison of the respective values of λ (Table V).

Thus the bucks give the more accurate result in a vitamin A test, while the does give a slightly more accurate result in a vitamin D test.

Table V. *The relative degrees of accuracy obtainable by the growth method for vitamins A and D.*

	Bucks		Does	
	Vitamin A	Vitamin D	Vitamin A	Vitamin D
λ	0·293	0·526	0·402	0·385
λ^2	0·086	0·277	0·162	0·148

Comparison of this method with the line test for the routine testing of samples for vitamin D.

The growth method and the line test appear to give the same results in the estimation of vitamin D. The line test appears to us the more useful for routine testing, as the preparatory period is limited to 3–3½ weeks and the test period to 10 days. In the growth method the preparatory period may be 1–3 months, the test period is 5 weeks, and the point at which a rat is ready for a test is less certain, *i.e.* one has to wait for 2–3 weeks in order to be sure that a rat has ceased to grow (*cf.* 10 days for a vitamin A test). The growth method is decidedly more tedious than the line test.

Discussion.

Two points of biological interest emerge from this work. The first is noticeable in the behaviour of the animals on the basal diet, and also in their response to doses of vitamin D. Young rats on the vitamin D-free diet grow more slowly than rats on a similar vitamin A-free diet. Also, the rats on the vitamin D-free diet do not rapidly lose weight and die when their reserves of D are presumably exhausted (as evidenced by the cessation of growth). They often maintain weight for many weeks before they die. Rats on a vitamin A-deficient diet, on the other hand, seldom remain steady in weight for more than 10 days when their reserves of vitamin A are exhausted (as evidenced by their cessation of growth). They lose weight rapidly and die. Does this mean that the animal's need for vitamin D is less than its need for vitamin A? The response to doses of vitamin D is also considerably less than to doses of vitamin A, which is indicated by the slopes of the curves connecting response to dose of vitamin given. For bucks the slopes are given by the figures 50·3 for vitamin A and 30·03 for vitamin D, and for the does the slopes are given by the figures 27·4 for vitamin A and 22·74 for vitamin D. The second point of interest emerges from a comparison of the results from which the curves of response have been drawn with the results obtained in an earlier attempt to make such curves. The results of this previous experiment are collected in Table I C. It may be seen that the response to doses of vitamin D is consistently lower in the group of animals which received vitamin A as 0·04 mg. of the light petroleum-soluble fraction of dried carrots than in the group of animals which received vitamin A as 0·04 mg. crystalline carotene. If insufficient vitamin A were limiting the response of the animals

in Table I C one would expect that the lower doses of vitamin D in the two groups would produce equal responses, *i.e.* the shortage of vitamin D would limit the response equally in the two groups, and that the higher doses of vitamin D would only gain higher responses in the group having the larger amounts of vitamin A. But the influence of the shortage of vitamin A appears to be felt in the lower doses of D also. Does this indicate some inter-relationship between the effects of vitamins A and D?

Summary.

A method for the estimation of vitamin D by means of its growth-promoting power has been worked out on similar lines to that proposed for vitamin A.

The responses of animals have been found to be graded to the dose of vitamin D given, that of the bucks being greater than that of the does. Equations (logarithmic) have been calculated to express the responses of the bucks and does respectively.

The degree of accuracy of this method of estimation of vitamin D is, by the use of bucks, only about one-third of that obtainable in a vitamin A test. By the use of does, the degree of accuracy of the vitamin D test is slightly greater than that of a vitamin A test.

The degree of accuracy obtainable by the line test in this laboratory has not yet been worked out. Tests of the same substances by the line test and by the growth method have given identical results. The growth method is, therefore, thought to measure the same factor as the line test. It is not however, as quick a method as the line test, and it is highly improbable that its degree of accuracy is any greater than that of the line test. It is therefore not recommended for routine testing.

Incidentally, the results reported here seem to indicate that the rat's need for vitamin A is greater than its need for vitamin D.

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CLXXXIX. THE DETERMINATION OF VITAMIN A IN COD-LIVER OILS, (A) BIOLOGICALLY, (B) CHEMICALLY, (C) PHYSICALLY, WITH A STATISTICAL EXAMINATION OF THE RESULTS.

II. FURTHER EVIDENCE THAT THE INTENSITY OF ABSORPTION AT 328$m\mu$ GIVES THE BEST AGREEMENT WITH THE BIOLOGICAL MEASURE OF VITAMIN A IN COD-LIVER OILS.

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(*Received August 20th, 1932.*)

In a previous paper [Coward, Dyer *et al.*, 1931] it was shown from an examination of eleven samples of cod-liver oil and two concentrates that, taking into account the known sampling error of the biological test, the measurement of the absorption by the oil at 328$m\mu$ gave the best agreement with the vitamin A value as determined by a biological test. The "blue value" of the unsaponifiable fraction of the oil gave rather less good agreement, while the "blue value" of the oil itself gave only a very crude measure of the biological activity. It was pointed out, however, that the intensity of absorption of certain oils lay outside the range of the possible biological value. In particular it was noted that two of these oils, C and G, having the same biological values (155 and 153) had absorption bands whose relative intensities were 103 and 257 respectively, and it was suggested that some unknown factor might have influenced the biological test and invalidated the result. Moreover the results of all the tests were evaluated by means of the curve drawn up in these laboratories by Coward, Key, Dyer and Morgan [1930; 1931] and expressed in terms of the oil A originally used for the making of the curve, without making a simultaneous test on this oil at the time of examination of oils C and G. The values obtained would be true in relation to oil A and to each other, only if the whole stock of rats was not varying in sensitivity during this time. As we had no information on this point, it was decided to test oils C and G again making a simultaneous test on oil A and using "litter-mate" animals in the three groups. This was done

twice, in June–July and in August, respectively, of 1931. In the August experiment, a re-test of oil L (of very high vitamin content) was also included. The agreement between oils C and G in biological value was confirmed but when the intensity of absorption at 328 $m\mu$ of oil C was re-examined it was found to have fallen about 20 %, and that of oil G to have fallen about 50 %. Thus the intensity of the 328 $m\mu$ band of oil G was only about 150 % of that of oil C. Tests on five fresh samples of cod-liver oil, each one with a simultaneous test on oil A, have also been made during the last nine months. Chemical and physical tests have been made on all the oils at the same time as the biological tests. The whole of the new tests on the old oils and the tests on the new samples have been collected and statistical examinations of the results have been made by the same method as was employed in the previous paper.

It is evident that in this series of oils there is less good agreement between the physical and the biological tests than in the previous series, the range of value ($\pm 2\lambda$) allowed being that shown by Coward [1932] to hold for this colony of rats. It is practically the same as that calculated from a smaller number of tests in the previous paper by Coward, Dyer, Morton and Gaddum [1931].

Re-examination of oils C and G.

Comparisons of these oils were made by the method employed in the earlier examinations, though with the added precaution of dividing each litter of rats between the different groups used for testing the different oils. In the first test (June–July, 1931) on oils, A, C and G, the doses chosen proved to be somewhat low and a second test was made using higher doses. The potencies of oils C and G were calculated relative to the contemporary values of oil A (Table I). They again appeared to be equal in vitamin A value, the finding reported in the previous paper being thus confirmed.

Table I. *Re-examination of oils C, G and L in comparison with simultaneous test on oil A.*

Oil	Date of test 1931	Dose mg.	No. of ♂ rats on test	Av. incr. in wt. in 5 wks. g.	$\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$	Abscissa of curve of response corresponding to this increase	No. of ♀ rats on test	Av. incr. in wt. in 5 wks. g.	$\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$	Abscissa of curve of response corresponding to this increase	Av. abscissa of curves of response for bucks and does	Vitamin A potency (%) relative to oil A (contemporary test)	
A	June–July	2	9	11·4	18·55	1·0	4	9·25	19·98	0·77	0·93	100	
	Aug.	2	6	9·3	13·58	0·9	6	14·5	17·09	1·2	1·05	100	
C	June–July	1	4	−2·5	7·04	0·53	6	−2·8	9·86	0·28	0·38	82	Weighted mean =
	Aug.	3	4	27·7	8·43	2·12	5	18·4	7·38	1·65	1·86	118	101
G	June–July	1	8	−3·6	19·78	0·51	8	4·0	8·87	0·51	0·51	110	Weighted mean =
	Aug.	3	5	18·0	13·64	1·36	5	11·4	7·60	0·92	1·14	72	92
L	Aug.	0·1	6	−0·3	7·56	0·59	5	0·6	9·06	0·37	0·49	933	

Apparent deterioration of vitamin A value of oils A, C, G and L.

From column 12 of Table I it may be seen that a dose of 2 mg. of oil A produced, in June–July, 1931, a mean increase in weight in the rats equal to that which would have been brought about by 0·93 mg. when the curves relating increase of weight to dose of oil A were made. In August, 1931, 2 mg. of oil A appeared to be equivalent to 1·05 mg. of original value. Thus oil A seemed to have lost about half its vitamin A potency. Similarly oils C, G and L all seemed to have lost two-thirds of their original potency, which made it appear in August, 1931, that oils A, C and G were equal in vitamin A value and oil L was only nine times as potent as oil A, whereas, originally oils C and G had 150 % and oil L had 1560 % of the vitamin A value of oil A.

It is quite possible, however, that a difference in sensitivity of the rats accounted for these apparent decreases in vitamin A value. We have some information on this point from the successive tests on oil A which have been made for comparison with other oils (see later part of paper) but until we have more information, we consider it safer to make a simultaneous test on the standard oil with each test of a fresh sample. More satisfactory evidence on this point will be available if carotene proves to be a suitable standard of reference for vitamin A tests. Meanwhile, we have confined our attention to the relation between the biological (vitamin A) values of the oils and their colorimetric and spectrometric values as determined at the same time as the biological value.

Correlation between the biological, colorimetric and spectrometric values of oils A, C, G and L at different times.

In Table II are collected the various values of oils A, C, G and L, each biological value being given relative to that of oil A (= 100) as determined by a simultaneous test on that oil. The chemical and physical measurements were made by the same methods as were described in the previous paper.

Oil A has proved to be very constant in chemical and physical values and oils C, G and L show losses in intensity of absorption at $328 m\mu$ reconcilable with their losses of vitamin A potency relative to oil A. But the fact remains that oils C and G had equal vitamin A potency in 1930 when the intensity of absorption at $328 m\mu$ of oil C was about 2/5 of that of oil G, and they still had equal vitamin A potency in 1931 when the intensity of absorption at $328 m\mu$ of oil C was 3/5 of that of oil G. It seems impossible to avoid the conclusion that the measurement of the intensity of absorption at $328 m\mu$ has failed to measure the potency of oils C and G.

Oil G has shown curious behaviour in its colour tests also. The "blue value" of the oil itself has fallen to about two-thirds of its former value, the "blue value" of the unsaponifiable fraction to about two-fifths. The "blue values" measured spectrophotometrically have also shown serious falls. Oil L showed a

Table II.

Oil	Date of test	Biological value relative to oil A (=100) simultaneous test	Blue value, $SbCl_3$ test: Lovibond measure: Oil	Blue value, $SbCl_3$ test: Lovibond measure: Unsap. fraction	Blue value, $SbCl_3$ test: Spectrophotometric: 572 $m\mu$	Blue value, $SbCl_3$ test: Spectrophotometric: 606 $m\mu$	Ultraviolet band 328 $m\mu$; oil itself
A	Nov. 1929–May 1930	100	11·6	—	—	—	—
	Dec. 1930	—	9·0	17·25	0·70	0·92	0·78
	Mar. 1931	—	9·0	17·1	—	—	—
	Apr. 1931	—	—	—	0·75	1·04	0·80
	June–July, 1931	(100)	—	—	—	—	—
	Aug. 1931	(100)	—	—	—	—	—
	Sept. 1931	—	—	17·25	0·72	0·93	0·82
	Oct. 1931	—	12·2	—	—	—	—
	Dec. 1931	—	10·5	—	—	—	—
C	Mar. 1930	147	—	—	—	—	—
	Apr. 1930	180 } 155	—	—	—	—	—
	July 1930	202	—	—	—	—	—
	Oct. 1930	90	—	—	—	—	—
	Dec. 1930	—	7·6	12·9	0·50	0·61	0·67
	June–July, 1931	82 } 100	—	—	—	—	—
	Aug. 1931	118	—	—	—	—	—
	Sept. 1931	—	—	11·9	0·46	0·64	0·52
	Oct. 1931	—	8·5	—	—	—	—
G	Sept. 1930	153	—	—	—	—	—
	Dec. 1930	—	—	—	1·45	1·92	1·68
	Jan. 1931	—	28·0	42·0	—	—	—
	June–July, 1931	110 } 91	—	19·6	—	—	—
	Aug. 1931	72	—	—	—	—	—
	Sept. 1931	—	—	16·4	1·05	1·26	0·87
	Oct. 1931	—	10·1	—	—	—	—
L	Jan. 1931	1562	305·0	—	—	—	—
	Apr. 1931	—	—	266	7·5	12·15	8·0
	Sept. 1931	933	—	236	6·7	11·0	6·1
	Oct. 1931	—	197·0	—		—	—

fall of about 30 % in the blue value of the oil itself, but practically none in that of the unsaponifiable fraction. The colour values of oil C have not appreciably changed. The fluctuations in the "blue value" of oil A are indicative of the differences that may be obtained even by the same workers under supposedly standardised conditions.

It is evident that the chemical and physical values are not always measurements of the same substance.

A comparison between the recently obtained biological, colorimetric and

Table III. *Showing the results of the different tests all reduced to the same units, and the probable limits of error of the biological test (probability 21/22).*

Oil	Biological value: Most probable	Biological value: Possible range ($\pm 2\lambda$)	Blue value, $SbCl_3$ test: Lovibond measure: Oil	Blue value, $SbCl_3$ test: Lovibond measure: Unsap. fraction	Blue value, $SbCl_3$ test: Spectrophotometric: 572 $m\mu$	Blue value, $SbCl_3$ test: Spectrophotometric: 606 $m\mu$	Ultraviolet band 328 $m\mu$; oil itself
	I		II	III	IV	V	VI
A	100	69–144	97	98	99	93	114
C	100	66–153	68	68	63	64	72
G	90	62–130	81	93	144	126	121
L	930	534–1620	1572	1345	922	1103	846
Factors by which readings in Table II were multiplied to obtain the above results			7·98	5·70	137·6	100·3	138·7

Table IV. *Showing estimates of the logarithmic variance of the different tests.*

		Blue value, $SbCl_3$ test				
		Lovibond measure		Spectrophotometric		Ultra-violet band $328 m\mu$; oil itself
	Biol. value	Oil	Unsap. fraction	$572 m\mu$	$606 m\mu$	
	I	II	III	IV	V	VI
Biological test λ_1^2	0·0089	—	—	—	—	—
Ratio of biol./phys. results λ^2	—	0·0276	0·0189	0·0272	0·0238	0·0137
Phys. results $\lambda^2 - \lambda_1^2$	—	0·0187	0·0100	0·0183	0·0149	0·0048
Percentage range corresponding to $\pm 2\sqrt{\lambda^2 - \lambda_1^2}$ within which the result may be expected to lie 21/22 times	65–154	53–188	63–158	54–186	57–175	73–137

spectrometric values of these oils has been made by the same method as in the previous paper. It is summarised in Tables III and IV. It is evident from the values of λ (the logarithmic variance of the different tests) that again the $328 m\mu$ band gives the best agreement with the biological value and the blue value of the unsaponifiable fraction of the oil gives the next best measurement, though this is not as near to the $328 m\mu$ measurement as in the last series of oils.

Examination of five fresh samples of cod-liver oil.

Five further samples of cod-liver oil have been examined by these methods, each one in comparison with oil A by a simultaneous test on that oil. The results are collected in Table V and analysed in Tables VI and VII. In Table

Table V. *Biological, chemical and physical measurement of five fresh samples of cod-liver oil, each biological value being expressed as a percentage of the biological value of oil A as determined by a simultaneous test on "litter-mate" animals.*

				Blue value, $SbCl_3$ test				
	No. of rats on test		Biol. value relative to oil A	Lovibond measure		Spectrophotometric		Ultra-violet band $328 m\mu$; oil itself
Oil	♂	♀	(=100)	Oil	Unsap. fraction	$572 m\mu$	$606 m\mu$	
R	5	5	570	17·1	32·7	1·07	1·26	0·65
S	7	3	314	23·0	42·8	1·24	1·46	0·75
T	5	4	440	19·6	25·5	0·92	0·94	0·60
U	9	0	314	23·7	34·3	1·10	1·34	0·67
V	4	6	532	13·1	19·8	0·61	0·81	0·52

VI it may be seen that the $328 m\mu$ measure of each of the oils falls within the range of the biological test though in three of the oils it only just does so. It is obviously a rather better measure of the biological value than either of the other measures. In Table VII it is again seen from the values of λ that the $328 m\mu$ measure is the best measure of the biological value. The other measures seem to be of approximately equal, but low, value.

Table VI. *Showing the results of tests on oils R, S, T, U, V, all reduced to the same units and the probable limits of error of the biological test (probability 21/22).*

	Biological value		Blue value, $SbCl_3$ test				
			Lovibond measure		Spectrophotometric		Ultra-violet $328m\mu$; oil itself
Oil	Most probable	Possible range	Oil	Unsap. fraction	$572m\mu$	$606m\mu$	
S	314	195–506	513	599	538	541	498
U	314	216–456	529	480	477	497	445
T	440	257–753	437	357	399	349	398
V	532	316–895	282	277	265	300	345
R	570	343–948	311	458	464	467	432
Factors by which readings in Table V were multiplied to obtain the above results			22·3	14·0	433·7	370·8	664·0

Table VII. *Showing estimates of the logarithmic variance of the different tests on oils R, S, T, U, V.*

		Blue value, $SbCl_3$ test				
		Lovibond measure		Spectrophotometric		Ultra-violet band $328m\mu$; oil itself
	Biological value	Oil	Unsap. fraction	$572m\mu$	$606m\mu$	
Biological test λ_1^2	0·01281	—	—	—	—	—
Ratio of biol./phys. test λ^2	—	0·04870	0·05250	0·04442	0·04375	0·02865
Phys. result $\lambda^2-\lambda_1^2$	—	0·03589	0·03969	0·03161	0·03094	0·01584
Percentage range corresponding to $\pm 2\sqrt{\lambda^2-\lambda_1^2}$ within which the result may be expected to lie 21/22 times	59–168	42–239	40–250	44–227	44–225	56–179

Discussion.

By making estimations of the vitamin A content of cod-liver oils by comparison with a simultaneous test on a standard oil of reference, it has been shown that the $328m\mu$ band of the oil gives the best agreement with the biological value. The "blue values" obtained by the action of antimony trichloride on the oil itself, or on the unsaponifiable fraction of the oil, give less good agreement. The $328m\mu$ values of some of the oils recently tested, however, only just fall within the range of possible biological values. It was thought to be at least possible that the method of estimating a sample of oil in terms of the value of the standard determined by a simultaneous test made on a reasonable number of animals (ten to twelve) might introduce a further error; for the value of the standard found in that particular test might be somewhat low owing to the normal variation in the response of the animals, whereas the value of the unknown might, for the same reason, be high. On the other hand, the normal variation of the animals might give a high value for the standard and a low one for the unknown. Then the value of the unknown would appear to be much less in the second event than in the first. The actual variation found

in testing the standard seven times showed that it might have consequences of the kind suggested. The seven values for the standard oil found during ten months were:

Date of test	Biological value: Most probable	Biological value: Range $\pm 2\lambda$	No. of animals used: ♂	No. of animals used: ♀
June–July, 1931	46·5	31–71	9	4
August, 1931	52·5	33–83	6	6
October, 1931	59·0	38–89	7	7
November, 1931	36·7	23–58	6	6
Jan.–Feb. 1932	76·2	46–126	5	5
March, 1932	64·3	39–107	0	13
April–May, 1932	55·4	32–97	3	6
Weighted mean	55·8	46–67	36	47

The distribution of these values proved to be normal [Fisher, 1930].

$$\gamma_2 = -0{\cdot}58 \text{ (standard error} = 1{\cdot}85).$$
$$\gamma_1 = 0{\cdot}118 \text{ (standard error} = 0{\cdot}925).$$

Thus it seemed possible that we might get truer values for the vitamin content of oils by ignoring the fluctuations in results found in the simultaneous tests, and state the values of the oils in terms of the same (average) value of the standard oil.

This calculation has been made. A summary of it is given in Table VIII. When the values of λ are compared with those of λ in Table VII it is obvious that the "fit" of the $328 m\mu$ band with the biological value is distinctly less

Table VIII. *Estimates of the logarithmic variance of the tests on oils R, S, T, U, V, ignoring the fluctuations found in simultaneous tests of the standard oil.*

	Biological value	Blue value, $SbCl_3$ test: Lovibond measure: Oil	Blue value, $SbCl_3$ test: Lovibond measure: Unsap. fraction	Blue value, $SbCl_3$ test: Spectrophotometric: $572 m\mu$	Blue value, $SbCl_3$ test: Spectrophotometric: $606 m\mu$	Ultra-violet band $328 m\mu$; oil itself
Biol. test λ_1^2	0·01281	—	—	—	—	—
Ratio of biol./phys. test λ^2	—	0·06781	0·09032	0·07840	0·07850	0·05465
Phys. result $\lambda^2 - \lambda_1^2$	—	0·05500	0·07751	0·06559	0·06569	0·04184
Percentage range corresponding to $\pm 2\sqrt{\lambda^2 - \lambda_1^2}$ within which the result may be expected to lie 21/22 times	59–168	34–294	28–360	31–325	31–326	39–257

good when the average value of the standard is used for comparisons, than when a contemporary value of the standard is used for comparisons. It would, therefore, appear that we have evidence that in making an estimation of the vitamin A content of a substance, a simultaneous test on the standard should always be made.

Summary.

The re-examination of four oils and the examination of five further samples of cod-liver oil by the biological, chemical and physical methods already described in a previous paper indicate again that the intensity of absorption at

328 $m\mu$ by the oil itself gives the best measure of the vitamin A value of the oil.

In the re-testing of the old oils, the blue value of the unsaponifiable fraction again gave better agreement with the biological value of the oil than did the blue value of the oil itself, but in the tests made on freshly prepared oils, there was no evidence that any one method of measuring the blue value was better than the rest. They all gave approximately the same results.

Some evidence is given that a more accurate estimation of the vitamin A content of an unknown oil in relation to a given (standard) oil is obtained if a simultaneous test on the standard is made and the respective responses compared, than if the response to the unknown is compared with an average response of the standard.

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Note added 26th September, 1932. A. Chevallier (Marseille) reported at the Physiological Congress held in Rome (September, 1932) that the intensity of absorption at 328 $m\mu$ gave good agreement with the biological value only in oils having a low free fatty acid value; the free fatty acids showed strong absorption at 317 $m\mu$ which interfered with the measurement of the 328 $m\mu$ band. Thus the intensity of absorption at 328 $m\mu$ is reliable as a measure of the vitamin A content only in fresh oils, or in older oils having a low free fatty acid value.

CXC. OBSERVATIONS ON THE HEXOSE-MONOPHOSPHATE CONTENT OF MAMMALIAN MUSCLE.

I. EXPERIMENTS ON RATS INJECTED WITH ADRENALINE.

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Cori and Cori [1931, 1] have recently described a comparatively simple method for determining the hexosemonophosphate in rat muscle, and have shown by its use that injection both of adrenaline and insulin raises the percentage therein contained [1931, 2]. The present paper records results confirming the accuracy of the Coris' method, as well as the effects of adrenaline injections in rats. It suggests certain modifications in the method of removal of the muscle, and in the carrying out of the early stages of the isolation of the hexosephosphate.

The method of Cori and Cori consists essentially in preparing a trichloroacetic acid extract of muscle removed by blunt dissection from the living animal. By a process involving a p_H adjustment by the addition of barium hydroxide to the extract, whereby inorganic phosphate is eliminated, followed by precipitation by ammoniacal alcohol, the barium hexosephosphate is isolated free from all but minute traces of interfering reducing substances, and contaminated by only small amounts of inorganic phosphate. In carrying out the analyses the original method was followed in strict detail, with one exception, namely that the first barium precipitate obtained on p_H adjustment was redissolved in 1 cc. of 10 % trichloroacetic acid and 4 cc. water, instead of being treated with sulphuric acid, prior to the second precipitation[1].

The reducing power of the final product is estimated by the Hagedorn-Jensen method (checked against a standard solution of glucose), and total phosphorus and inorganic phosphorus are determined by the procedure of Fiske and Subarrow. The reducing power (Hagedorn-Jensen) multiplied by 1·5 gives the reducing power expressed as hexose, and this value divided by 5·8 gives the theoretical phosphorus content.

The amounts of organic phosphorus found agreed closely with the theoretical values calculated from the reduction observed, as will be seen from Table I, containing 25 results, selected at random from over 100 estimations.

[1] For suggesting this modification, which yielded, in the author's hands, much more consistent results, thanks are due to Dr P. Eggleton, of the University of Edinburgh.

Table I. *Estimation of hexosemonophosphate.*

The results are expressed in mg. per 100 g. of tissue.

Hexose	Phosphorus (calculated)	Organic phosphorus (found)
27·5	4·8	5·2
32·0	5·5	6·7
36·7	6·3	6·0
40·6	6·9	7·0
41·5	6·9	5·6
42·4	7·3	5·1
47·0	8·1	5·8
47·6	8·2	9·1
47·8	8·2	6·7
48·3	8·3	8·3
50·6	8·7	9·4
58·8	10·1	9·9
62·2	10·2	10·5
62·4	10·7	12·1
62·6	10·7	8·9
64·1	11·1	11·1
65·8	11·3	11·1
67·6	11·6	10·8
68·3	11·7	12·4
68·8	11·8	11·2
72·7	12·6	13·9
74·7	12·9	14·0
91·7	15·8	14·4
128·0	22·0	21·7
165·9	28·6	28·7

Experiments employing blunt dissection of the muscle from the living animal.

The rats, fed on a standard diet, were anaesthetised by intraperitoneal injection of a 2 % amytal solution, dose 10 mg. per 100 g. body weight. Within a short period the animal showed no reflex on pinching the tail, and at a known time after administering the amytal, the gastrocnemius of one limb was removed by blunt dissection, as recommended in the original paper, and prepared for analysis. A number of experiments were carried out in which the muscle of the second limb was removed, after a known interval, without giving adrenaline. No change in the resting value of the hexosephosphate was found, even after anaesthesia lasting three hours. (See Table II.)

Table II. *Hexosemonophosphate content of rat gastrocnemius muscle under amytal anaesthesia.*

In this and the following tables, results are expressed as mg. hexose/100 g. tissue.

Exp.	Muscle removed from first limb, time after giving amytal (mins.)	Muscle removed from second limb, time after giving amytal (mins.)	Hexosephosphate limb I	Hexosephosphate limb II
1	15	30	53·5	62·3
2	15	30	55·5	60·7
3	30	120	53·6	51·9
4	60	90	51·2	50·2
5	120	180	45·2	42·0

Mean value for resting muscle 52·5.

Intraperitoneal injection of adrenaline (0·02 mg. per 100 g. body weight) brought about a rapid and marked increase in the hexosephosphate content of the muscle. The muscle from one limb was removed as a control, prior to administering the adrenaline. It will be seen from Table III that the values obtained in this group of experiments are closely similar to those of Cori and Cori.

Table III. *The influence of adrenaline on the hexosemonophosphate content of rat gastrocnemius muscle.*

Exp.	Muscle removed from second limb, time after giving adrenaline (mins.)	Hexosephosphate: Before adrenaline I	Hexosephosphate: After adrenaline II
1	15	58·6	71·9
2	15	49·3	71·9
3	15	36·3	70·3
4	15	50·0	70·1
5	30	36·3	99·1
6	30	57·5	97·9
7	30	46·6	96·1
8	30	44·1	98·0

Mean value for resting muscle 47·3.

The further observation that adrenaline causes an increase in the hexosephosphate, which rises to a maximum and then gradually returns to normal, was fully confirmed in a group of experiments in which adrenaline was given before removal of any muscle, one limb being dealt with early in the experiment, and the second limb after a considerable period had elapsed. (See Table IV.)

Table IV. *Experiments showing the return to normal of the hexosephosphate content of rat muscle after adrenaline injection.*

Exp.	Times after adrenaline injection, muscles removed from limbs (mins.): I	Times after adrenaline injection, muscles removed from limbs (mins.): II	Hexosephosphate: I	Hexosephosphate: II
1	30	120	84·2	60·6
2	30	180	91·7	50·6
3	60	120	128·0	62·6

The close correspondence between the results obtained in the present work, and those of Cori and Cori is emphasised by the results shown in Table V.

Table V.

Results are means derived from the different groups of experiments.

Time after adrenaline (mins.)	0	15	30	60	120	180	240
Hexosephosphate: Bell	49·3	71·1	94·5	128·0	61·1	50·6	—
Hexosphosphate: Cori	53·3	88·0	86·9	108·6	72·8	66·3	54·0

Experiments employing rapid amputation of the whole limb before dissection of the muscle.

As the dissection of muscle from the larger laboratory animals, such as cats and rabbits, is not advisable from the point of view of obtaining it as far as possible undisturbed, the effect of rapid amputation and freezing of the whole limb, as described by Cleghorn and Peterson [1932] was investigated. These workers used a chopper and mallet to remove the limbs of cats for glycogen estimation, but in the present work it was found that sharp, heavy shears were convenient for effecting rapid amputation. Control experiments, in which the effect of amytal alone was studied showed that this method gave results comparable with the dissection method, provided the muscle did not twitch (Table VI).

Table VI. *Experiments in which the whole limb was removed and frozen before muscle was taken for analysis. No adrenaline was given.*

Exp.	Time elapsing between removal of the two limbs (mins.)	Hexosephosphate I	Hexosephosphate II
1*	60	73·5†	59·6
2*	60	49·2	55·0
3*	60	78·8†	64·3
4	30	66·0	78·5†
5	30	61·3	57·9
6	30	57·1	57·8

Mean value for resting muscle († excepted) 58·7.

* In these experiments the second limb was not amputated, the muscles being removed by blunt dissection as in the previous experiments.
† Muscle twitched on amputation of limb.

The effect of adrenaline injection was investigated by this method and was found to lead to results strictly comparable with those of the dissection method, again provided that the muscle did not twitch (Table VII).

Table VII. *Action of adrenaline. Both limbs removed by amputation.*

Exp.	Time elapsing between giving adrenaline and amputation of second limb (mins.)	Hexosephosphate I	Hexosephosphate II
1	15	121·7*	129·0*
2	15	41·8	71·1
3	15	39·9	74·6
4	15	58·0	74·8
5	30	48·3	110·1

Mean value for resting muscle (* excepted) 49·5.

* Muscle twitched.

Remarks on technique.

When the experiments were prolonged beyond an hour's anaesthesia, the rats were tracheotomised and supplied with oxygen in order to guard against the possibility of anoxaemia, since it was found that so great a secretion of mucous took place as sometimes to lead to death of the animal by obstruction of the trachea.

In certain experiments, notably in 3 and 5, Table III, and 2 and 3, Table VII, the values of hexosephosphate found in the first limb were rather below the normal range. In these instances, considerable amounts of inorganic sulphate were found in the muscle extracts. Possibly the barium sulphate formed on p_H adjustment had exerted its well-known power of adsorption and had carried down with it a small fraction of the barium hexosephosphate. The occurrence of this inorganic sulphate was not noticeable except recently after induction of anaesthesia, and it is here suggested that its appearance may be connected with the action of a detoxicating mechanism.

SUMMARY.

The action of adrenaline on the hexosemonophosphate content of rat muscle has been investigated by the method of Cori and Cori, and the results of these workers have been fully confirmed. Amputation and freezing of the whole limb of the animal, prior to removal of the muscle for analysis, has been shown to yield equally satisfactory results with the method of dissection from the living animal.

The author is indebted to Prof. J. J. R. Macleod for the interest which he gave to this work.

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CXCI. METABOLISM IN TETANY.

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In the communication of Greenwald [1932] criticising the work detailed in the paper on metabolism in tetany by Morris, Watson and Morris [1931] the data to which most objection is taken are those referring to the phosphorus and calcium intake. Greenwald states that our ratios for the combined oatmeal milk diets are with three exceptions lower than the usual P/Ca for milk which he gives as 1·7. But according to Sherman the phosphorus and calcium contents of whole milk are 0·093 % and 0·120 % respectively, giving a P/Ca ratio of 0·78. It is clear that Greenwald is using the $\frac{P_2O_5}{Ca}$ ratio which according to Sherman is 1·77. In our paper we stated definitely that the mineral intake was computed as calcium and phosphorus. It would appear that this mistake of Greenwald in assuming a ratio of 1·7 for the P/Ca ratio invalidates his criticism of our calcium and phosphorus figures, the variations in which are clearly due to the varying amounts of milk and oatmeal consumed.

In the analyses sent to Greenwald privately and quoted by him the figures for phosphorus are obviously those used for P_2O_5 so that in these particular milks (not those used in the tetany experiments, which were "separated") the P/Ca ratios varied from 1·1 to 1·2.

As regards the difficulty with the nitrogen-chlorine ratios it is stated on p. 789 [1931] that the cats were treated with saline. Protocols had been prepared giving the exact amounts of NaCl administered and the times of administration. Unfortunately for reasons of space-economy they had to be omitted, but we did not feel this was of great import since all the chlorine injected during each period was included in the intake for that period. This explains Greenwald's difficulty in co-ordinating the ratios. The porridge-milk mixture was sampled every day and analysed: in addition all the food residues were collected and analysed daily. Table I shows the nitrogen-chlorine ratios for the cat-feeds in all the periods. The variation in the normal periods except for that of Cat 4 is not very great when it is remembered that no attempt was made to weigh the quantity of salt added. This was obviously unnecessary since the food mixture itself was analysed. If we estimate the intake of Cl during the active period of Cat 3 (quoted by Greenwald) on the assumption that the N/Cl ratio

Table I.

Nitrogen/chlorine ratios of the food-intake in the various periods.

Cat	I.	Normal	4·4
		Latent	4·5
		Active	3·7
		Recovery	4·1
„	II.	Normal	6·2
		Latent	5·7
„	III.	Normal	5·5
		Latent	2·6
		Active	1·0
		Recovery	5·5
„	IV.	Normal	8·8
		Latent	5·7
„	V.	Normal	6·0
		Latent	4·8

of the food was the same as that for the normal period we find that it should be about 0·130 g.; leaving, according to our figure, 0·546 g. of Cl to be injected. Actually 20 cc. of $M/2$ NaCl was injected, *i.e.* 0·355 g. of Cl—a close enough approximation if one remembers that the oatmeal and milk were consumed in different proportions during the two periods.

Greenwald complains of the considerable variation in the compositions of the milk and oatmeal. As already stated we did not analyse samples of the individual foodstuffs for each period but only the actual food mixtures and residues daily. Nevertheless Table II shows how close the figures are when we

Table II.

	N	Ca	P
I. Oatmeal 100 g.	3·3	0·100	0·200
Milk 100 g.	0·63	0·120	0·120
Total	3·93	0·220	0·320
Amount actually consumed by Cat III. Normal period	3·90	0·203	0·293
II. Oatmeal 12 g.	0·40	0·012	0·024
Milk 50 g.	0·32	0·060	0·060
Total	0·72	0·072	0·084
Amount actually consumed by Cat III. Active period	0·71	0·057	0·110

assume a certain ingestion of oatmeal and milk and use the analytical results of samples of oatmeal and milk obtained before the commencement of the experiments.

The high retentions of nitrogen in Cats 3, 4 and 5 are commented on. It need only be said that the cats which were young, were imported into the laboratory just prior to the experiment, that their previous feeding was unknown and that the nitrogen figures do not enter into the argument of the paper.

Finally we regret the mistake in giving the date of publication, and the error in writing "decreased" instead of "increased" in the first line of the paragraph headed "Phosphorus" on p. 795. As regards the interpretation of conclusions we feel that we were just as entitled to draw these deductions from Greenwald's paper as he is entitled to criticise our own data.

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CXCII. THE NATURE AND COMPOSITION OF THE MUCILAGE OF THE SEED OF WHITE MUSTARD (*BRASSICA ALBA*).

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In comparison with the immense bibliographies of starch and cellulose for example, the literature of the gums and mucilages is scanty. This, doubtless, is due in part to the great commercial importance of the former, but a contributory cause may be sought in the practical difficulties involved in dealing with the latter. The gums and mucilages are complex colloids which are not infrequently found to be mixtures of almost equally complex substances of closely similar physical and chemical properties. Separation of the constituents is consequently difficult, nor, when a separation has been achieved, is the characterisation of the components an easy matter. Nevertheless, increased knowledge of the chemical nature of such substances should be of interest to the plant biochemist and physiologist alike, and it is conceivable that the industrialist may also benefit ultimately as a result of such studies.

Comparatively modern researches on the plant mucilages may be said to date from the end of the nineteenth century, when Gans and Tollens [1888] investigated the mucilage of quince. Similar products were examined by Yoshimura [1895] and von Bitto [1895], and this work was supplemented later by investigations of the mucilage of the prickly pear [Harlay, 1902], and of linseed [Hilger, 1903; Neville, 1913].

The early researches were concerned chiefly with the nature of the sugars produced on hydrolysis, and in the case of linseed mucilage, for example, Hilger reported xylose, glucose, arabinose and galactose as constituent sugars. Neville also isolated the "barium salt of an organic acid," but was not convinced that the acid was a constituent of the mucilage, regarding it as an impurity. This observation is of peculiar interest in view of the fact that the existence of uronic acids in such plant materials was at that time unsuspected.

Re-awakened interest in the study of such plant products as pectin, hemicelluloses, gums and mucilages has followed the recognition of the wide occurrence of these acids, and recent investigations tend further to emphasise their importance. Galacturonic acid had been shown to be a constituent of

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pectin by Ehrlich [1917], and the occurrence of this acid and of glycuronic acid in various hemicelluloses was also demonstrated [O'Dwyer, 1926; Norris and Preece, 1930, and others]. Another member of the class, mannuronic acid, whose existence in plant products was foreshadowed by Cretcher and Nelson [1929] was shown to be a constituent of the cell-wall substances of *Laminaria* spp. by Bird and Haas [1931].

There is now reason to believe that in many plant products the uronic acid forms an aldobionic acid nucleus which is relatively resistant to hydrolysis. Thus, Butler and Cretcher [1929] isolated from gum arabic a galactose-glycuronic acid, and Anderson and Crowder [1930] prepared rhamnose-galacturonic acid from linseed mucilage by similar methods. Again, the specific carbohydrate of pneumococcus, isolated by Heidelberger and Goebel [1927] appears to be a polymeride of glucose-glycuronic acid.

The object of the present investigation of the mucilage of mustard seed was not solely the identification of the products of hydrolysis, but involved also the separation of the product into definite fractions. The mode of fractionation, described later, was not based on physical properties—precipitability by alcohol, acetone, *etc.*—as in the case of the hemicelluloses [Norris and Preece, 1930; Preece, 1930], but was effected by more truly chemical means, namely the action of alkali. We have been able to show that the mucilage is composed of cellulose together with acid polysaccharides which yield arabinose, galactose, rhamnose, galacturonic acid and glycuronic acid on hydrolysis, and contain methoxyl groups. The term "acid polysaccharides" has been very generally used by American workers in this field, to indicate polysaccharides which yield uronic acids in addition to simple sugars on hydrolysis. The term "polyuronide" advocated by Schryver has much to recommend it, but does not appear to have found wide acceptance. However, in the present confusion of nomenclature, any attempt at uniformity is desirable, and in this paper the term acid polysaccharide will be used.

By analogy with pectin, it was at first assumed that the methoxyl groups were bound to the carboxyl groups of the uronic acids by ester linkages. This view was, however, abandoned when it was found that the former resisted saponification by alkali, and it must therefore be assumed that these methoxyl groups are bound by ether linkages. A similar phenomenon was observed by O'Dwyer [1928] in the hemicelluloses of beech and oak [see also von Fellenberg, 1918; Anderson and Otis, 1930].

Evidence has been adduced that the rhamnose and at least some of the galactose combines with the uronic acid to form stable aldobionic acid nuclei, similar to those cited by Butler and Cretcher [1929] and Anderson and Crowder [1930]. Such aldobionic acids survive, at least in part, the hydrolytic action of 4 % sulphuric acid at 100° for 16–20 hours.

On soaking the mustard seed in cold distilled water, an extract is obtained which contains the mucilage, precipitable by alcohol, and also free sugars, amongst which galactose was identified. If, then, to this extract an equal

volume of saturated barium hydroxide solution is added, a gel is immediately precipitated, and after standing overnight, may be filtered off. The filtrate contains the free sugars above-mentioned, and also a water-soluble product, Fraction I. This fraction is precipitable by alcohol, yields on hydrolysis rhamnose, arabinose, galactose and galacturonic acid, and contains methoxyl groups.

The gel obtained by the action of barium hydroxide may be further fractionated by the action of sodium hydroxide solution, which effects its separation into cellulose, Fraction II, and a sodium hydroxide-soluble product, Fraction III, precipitable by alcohol, which yields on hydrolysis arabinose, galactose, galacturonic acid and glycuronic acid but no rhamnose. This fraction also contains methoxyl groups. It was found impossible to free the cellulose from substances which gave a positive naphthoresorcinol reaction; such substances, containing uronic acid and furfuraldehyde-yielding groups, may constitute an integral part of the cellulose itself, which may be of the nature of an oxycellulose; or the positive reaction may merely indicate that it is impossible to separate Fraction III completely.

All samples of the mucilage extract gave a strong positive reaction with benzidine and hydrogen peroxide, indicating the presence of peroxidase. In a histological examination of the testa, the sclerenchymatous palisade cells were found to give a similar strong peroxidase reaction, and these cells are only separated from the mucilage-containing cells of the cuticle by two rows of thin-walled collenchymatous cells. The possible significance of these observations is discussed later.

Experimental.

Preparation of the mucilage.

The dry seed is extracted with cold distilled water in the proportions of one part of seed to ten parts of water. The extraction is continued for 24 hours with efficient stirring or shaking, and the viscous extract obtained is filtered through muslin, while the seed is returned to the vessel for two additional extractions. The united extracts are then filtered through a bed of glass wool and muslin to remove foreign particles and the filtrate poured into alcohol, which precipitates a fibrous gelatinous product. Filtration, re-solution and reprecipitation of the mucilage may be carried out several times, the product then being dried in successive increasing concentrations of alcohol, and finally *in vacuo* over phosphorus pentoxide. Prepared thus, a snow-white fibrous product is obtained, corresponding in yield to about 2 % of the dry seed.

The mucilage swells in water and finally dissolves, giving an opalescent colloidal solution which gives no coloration with iodine, neither does it reduce Fehling's solution.

On heating the mucilage for a short time with dilute sulphuric acid a gel separated out, and was shown later to consist of cellulose. After more prolonged

hydrolysis and neutralisation with barium carbonate, barium salts of aldobionic acids, one of which contained rhamnose, were obtained. The residual solution after alcoholic precipitation of the barium salts, was shown to contain free sugars, namely, arabinose and galactose. The characterisation of these products is discussed in detail at a later stage.

Analysis of the mucilage.

Preliminary experiments had thus shown that the mucilage was composed of cellulose, together with compounds which contained carbohydrate and uronic acid residues. Analysis of the mucilage was then undertaken, the following determinations being made:

(i) Ash.
(ii) Cellulose.
(iii) Methoxyl.
(iv) Furfuraldehyde and methylfurfuraldehyde.
(v) Uronic anhydride.
(vi) Nitrogen (Kjeldahl).

Ash was determined by direct ignition at a dull red heat. Cellulose was determined by the method of Cross and Bevan [1889], with slight modifications [Norman, 1929, 2] chiefly designed to minimise manipulative losses. Methoxyl was determined by the Perkin [1903] modification of Zeisel's method. Furfuraldehyde and methylfurfuraldehyde were estimated by the phloroglucinol methods of Tollens and Kröber, and of Ellett and Meyer [Browne, 1912]. The probable inaccuracies involved in these estimations need no emphasis at this stage; the chief source of error in the estimation of methylfurfuraldehyde in the present instance is the presence of ω-hydroxymethylfurfuraldehyde from hexosic material, the results being somewhat high on this account. Uronic anhydride was estimated by the decarboxylation method of Nanji, Paton and Ling [1925], using a modified apparatus which gave accurate results with less than 0·1 g. of material.

Ash. Specimens of mucilage, prepared from different samples of seeds under slightly varying conditions, yielded varying quantities of ash. After dialysis of the mucilage in thin collodion bags of high permeability for 20–40 days, the ash in all cases fell to about 3·5 %.

Ash content of samples of mucilage.

Sample	Original ash %	After 20–40 days' dialysis: ash %
A	8·4	3·75
B	6·82	3·38
C	6·16	3·60
D	5·73	3·57
E	16·2	—

The ash of the dialysed mucilage was submitted to spectrographic analysis: calcium, magnesium and iron were present in abundance; strontium and

aluminium were also found. Sodium and potassium were conspicuous by their absence. The presence of silicon and phosphorus was demonstrated by chemical means.

It is not improbable that the undialysable ash forms an integral part of the mucilage, which may exist naturally as a calcium, magnesium or iron salt; silicic and phosphoric esters also, are of common occurrence in complex polysaccharides, as exemplified in the case of starch and some hemicelluloses.

The different samples of mucilage showed variations in the analytical results obtained, not only in the case of the ash, but also more particularly with regard to the cellulose and methoxyl contents; the hexosan content (by difference) likewise reflected these variations. The most strikingly contrasted samples were C and E, which gave the following results, calculated on an ash-free basis.

Analysis of samples of mucilage.

	(%) C	(%) E
Cellulose	45·70	55·60
Methoxyl	2·00	1·43
Uronic anhydride	20·43	17·24
Pentosan	6·73	6·13
Methylpentosan	7·14	6·44
Hexosan (by difference)	18·00	13·16
Nitrogen	—	0·66

Separation of the mucilage into three fractions.

To a large volume of an aqueous extract of the mucilage, an equal volume of a saturated solution of barium hydroxide was added, when a gel very rapidly formed. After standing overnight in absence of air, the gel was filtered off through muslin and subsequently freed from liquid as far as possible in a mechanical press. If insufficient barium hydroxide is added the gel cannot be obtained in a fibrous condition and is then difficult to treat. After re-extracting twice with saturated barium hydroxide, the united filtrates and the residue were treated separately.

(*a*) *The filtrate.* The excess barium hydroxide in the filtrate was precipitated as barium carbonate by passing carbon dioxide into the solution. After filtration, the solution was evaporated under diminished pressure and from time to time carbon dioxide was again passed into the solution, the evaporation being continued after further filtration until the volume of liquid was considerably reduced. Alcohol to a concentration of 75 % was then added with rapid stirring, and after standing overnight a white flocculum was filtered off, washed with absolute alcohol and air-dried. The product was then redissolved in water, the solution saturated with carbon dioxide and allowed to stand overnight in the ice-chest. On subsequent heating for 30 minutes on the water-bath, a fine suspension of barium carbonate settled out and was removed at the centrifuge. The object of this procedure was to prevent "saccharate" formation. The clear solution was again poured into alcohol and the precipitate, Fraction I, was dried in the usual manner.

It may be mentioned in passing that the alcoholic filtrate from the above precipitation was concentrated, primarily with the object of recovering the alcohol. It was found that the liquid concentrated to a syrup which reduced Fehling's solution and contained free sugars. Similarly, a strongly reducing syrup may be obtained by concentrating the alcoholic filtrate from freshly precipitated mucilage extract. The free sugars appear to be extracted from the seed at the same time as the mucilage and are always present in the watery extract of the seed. As this observation was not germane to the main object of the research, the sugars were not thoroughly investigated, although one of them was identified as galactose. It should be noted, however, that if the fractionation of the mucilage is carried out with fresh mucilage extract, these sugars will be found in the alcoholic filtrate from Fraction I, but will not be present if a solution of precipitated mucilage is used as the starting-point.

(*b*) *The gel.* This, which was a complex of cellulose with acid polysaccharides, was separated into two components by extraction for four hours with 4 % sodium hydroxide solution at 40–50°, this treatment being repeated until the residual cellulose gave only the faintest naphthoresorcinol reaction. Usually three extractions sufficed, although it was found almost impossible to free the cellulose entirely from uronide constituents. The fibrous cellulose was treated with acid to remove free alkali and washed with water until free from acid and soluble inorganic salts. A white fibrous product was obtained on drying in the usual manner and was designated Fraction II.

After filtration of this fraction, the united sodium hydroxide extracts were neutralised with glacial acetic acid and poured into an equal volume of acetone. The white gum obtained was redissolved in 4 % sodium hydroxide solution, filtered through paper pulp and reprecipitated as a copper gel by addition of Fehling's solution. The copper gel was decomposed with hydrochloric acid and again precipitated with acetone. Re-solution and reprecipitation followed, until a white product, free from copper, was obtained. This was dried in the usual manner and constituted Fraction III.

Analysis of Fraction I.

Fraction I is a white powder which swells in water and finally dissolves; the solution gives no iodine coloration, nor does it reduce Fehling's solution. The specific rotation $[\alpha]_D^{15^\circ} = +10^\circ$.

On analysis by the usual methods, the following results were obtained:

	%
Barium	9·45
Pentosan	6·80
Methylpentosan	10·00
Uronic anhydride	24·20
Methoxyl	4·90
Hexosan (by difference)	44·65
Nitrogen	nil

Assuming all the barium to be combined with the available carboxyl groups of the uronic acid, this percentage—9·45—would correspond to 24·3 % of

uronic anhydride, a very close approximation to the figure obtained above by decarboxylation. That the carboxyl groups of the uronic acid are free to combine with barium in this manner appears also to be indicated by the fact that they are not in ester combination with methoxyl. Thus, on treatment for 2 hours with 2 % sodium hydroxide at 80°, the product still contained 3·5 % of methoxyl, showing that these groups are resistant to hydrolysis, and are therefore probably in ether-linkage [von Fellenberg, 1918]. Moreover, on prolonged dialysis of the product through cellophane membranes, only traces of barium passed out, and this is not likely to be the case unless the substance is to be regarded as a barium compound. The balance of evidence is thus in favour of this last conclusion.

Hydrolysis of Fraction I.

70 cc. of 2·5 % sulphuric acid were added to 3 g. of the product and the mixture was heated under reflux on a boiling water-bath for 4 hours. Neutralisation was effected by addition of saturated barium hydroxide solution, excess being removed by means of carbon dioxide. After filtration and evaporation under diminished pressure, the barium salt was precipitated by alcohol, and after drying weighed 0·9 g. The alcoholic filtrate was evaporated to dryness and the sugars extracted with successive portions of hot absolute, 80 % and 60 % alcohol. The fraction soluble in absolute alcohol contained a small amount of a sugar giving a positive orcinol reaction; the bulk of the sugar, however, dissolved in 60 % alcohol.

Fraction soluble in absolute alcohol. Arabinose was present, and was isolated as the characteristic diphenylhydrazone melting at 200–201°. Xylose was absent; in repeated attempts to prepare cadmium xylonobromide, fine needle-shaped crystals were obtained entirely different from the characteristic boat-shaped crystals obtained from authentic xylose.

Fraction soluble in 80 % alcohol. Galactose was present. The methylphenylhydrazone was prepared [van der Haar, 1920] and after several recrystallisations from 30 % alcohol, melted at 189°. This was confirmed by admixture with an authentic specimen of galactose methylphenylhydrazone; the mixture again melted at 189°.

Fraction soluble in 60 % alcohol. This consisted chiefly of galactose. On oxidation with nitric acid, sp. gr. 1·15, mucic acid was obtained, melting, after reprecipitation, at 219°.

The original syrup before extraction with alcohol gave negative results when tested for keto-hexoses by the Pinoff-Selivanoff method.

Treatment of the barium salt. The best means of identifying the methylpentose present in the fraction was afforded by an examination of the hydrolysis products of this barium salt. It has been stated that the methylpentose exists in relatively stable combination as an aldobionic acid and is therefore to be expected in the barium salt prepared as indicated. The barium salt was hydrolysed under more drastic conditions than in the original preparation as follows:

0·9 g. of the barium salt was boiled under reflux for 10 hours with 25 cc. of 5 % sulphuric acid, and after neutralisation with barium hydroxide, the solution was filtered, evaporated to a thin syrup and divided into two portions. After this treatment there was no precipitable barium in the syrup, indicating that the prolonged hydrolysis had broken up the aldobionic complex and destroyed the uronic acid as such. The only sugar likely to be present in the syrup was therefore the methylpentose, with, possibly a small amount of galactose. (The nature of the aldobionic acids is discussed on p. 1619.) The *p*-bromophenylosazone was prepared from the first portion of the syrup, according to the method indicated by van der Haar [1920]. The crude osazone was washed with ether followed by 70 % and then hot 95 % alcohol. There remained a yellow precipitate melting at 218°, and there was no depression of this melting-point on testing a mixture of the sample and an authentic specimen of rhamnose *p*-bromophenylosazone. The second portion of the syrup was freed from a trace of galactose by means of the methylphenylhydrazone, which was filtered off. Excess of methylphenylhydrazine was removed from the solution by shaking with ether, and the *p*-bromophenylosazone again prepared. This sample also melted at 218° and there is thus little doubt that the methylpentose in the barium salt is rhamnose.

Identification of the uronic acid. The method employed was that used by Heidelberger and Goebel [1927] in their study of the uronic acid from the specific polysaccharide of pneumococcus.

1·2 g. of Fraction I were dissolved in 12 cc. of 7·5 % hydrobromic acid containing 0·6 cc. of bromine, and heated on a boiling water-bath under reflux for 10 hours. The uronic acid produced on hydrolysis is immediately oxidised to a dicarboxylic acid, while free sugars are converted into the corresponding monocarboxylic acids. If, therefore, the dicarboxylic acid can be identified, the nature of the original uronic acid is known.

After standing for 24 hours a white precipitate was formed, which on purification weighed 0·06 g. and was found to be mucic acid, melting at 216°. The experiment was repeated with similar results and a control experiment with pure galactose yielded no trace of mucic acid. The original uronic acid from which the mucic acid was derived was galacturonic acid.

No other sugars or sugar acids could be detected by all the usual methods in the hydrolysis products of Fraction I, which therefore appears to consist of a barium salt or salts of polysaccharides which yield on hydrolysis rhamnose, arabinose, galactose and galacturonic acid, and also contain methoxyl groups. The plural is used advisedly, since there is no evidence that Fraction I is a definite chemical entity.

Analysis of Fraction II (cellulose).

This fraction was prepared from the mucilage not only by the alkali treatment outlined previously, but also more directly by acid hydrolysis as follows. An aqueous solution of mucilage was heated at the temperature of the boiling water-bath with 1–4 % sulphuric acid for 2 hours. At the end of this

period, a fibrous gel had separated and was filtered off, washed thoroughly until free from acid, dried in graded strengths of alcohol and finally to constant weight at 100°. The gel obtained by both the acid and alkali treatments was identified as cellulose by the following experiments.

(*a*) It was extraordinarily resistant to acid hydrolysis.

(*b*) It was soluble in concentrated zinc chloride solution.

(*c*) It gave a blue colour with chlor-zinc-iodine.

(*d*) 2 g. of the product were hydrolysed with 72 % sulphuric acid according to the method of Monier Williams [1921]. The only sugar present after hydrolysis was glucose as indicated by the characteristic phenylosazone, which melted sharply at 205°. The amount of glucose produced, as estimated by Fehling's method, corresponded to 80·5 % of the weight of gel taken, or 72·5 % of theory. The specific rotation of the purified sugar was $[\alpha]_D^{20^\circ} = +52{\cdot}0^\circ$ in a second hydrolysis using 10 g. of the product.

(*e*) A further 10 g. of the product were acetylated by the method of Irvine and Hirst [1922]. A pure white granular product was obtained, and the acetyl groups therein were estimated by the saponification method indicated in the above-quoted paper.

		%
Original cellulose prepared by acid treatment:	Found, CH_3CO	43·7
Original cellulose prepared by alkali treatment:	Found, CH_3CO	45·3
Theoretical for cellulose triacetate:	CH_3CO	44·8

The difference between the found and the theoretical figures is within the usual experimental error involved in such saponification methods, and hence it may be concluded that the acetate obtained is very closely similar to normal cellulose triacetate.

The cellulose obtained by alkali treatment was also subjected to analysis, with the following results:

	%	
Ash	13·30	
Furfuraldehyde	1·48	(ash-free basis)
Uronic anhydride	3·40	„

The presence of these substances would be responsible for the positive naphthoresorcinol reaction given by the cellulose, and the possible significance of this observation has been discussed previously.

Analysis of Fraction III.

Fraction III is a white powder, soluble in sodium hydroxide solution; it gives no coloration with iodine, nor does it reduce Fehling's solution.

The following analytical results, calculated on an ash-free basis, were obtained:

	%
Uronic anhydride	14·8
Pentosan	9·4
Methoxyl	3·4
Methylpentosan	nil
Nitrogen	nil
Hexosan (by difference)	72·4

It was impossible to determine the optical rotation owing to the highly opaque colloidal solution obtained even at considerable dilution.

Hydrolysis of Fraction III.

By similar methods (hydrolysis, preparation of substituted phenylhydrazine derivatives, *etc.*) to those already described for Fraction I, the following constituents were identified:

(*a*) arabinose (diphenylhydrazone, M.P. 201°).

(*b*) galactose (methylphenylhydrazone, M.P. 198°).

No other sugars were present, all the recognised tests giving negative results.

The characterisation of the uronic acids present in Fraction III calls for more detailed description. The method of procedure adopted was that of Heidelberger and Goebel [1927] previously described. In preliminary experiments using 1 g. and 3 g. respectively of the product, no mucic acid could be obtained, and it was at first thought that galacturonic acid was therefore not a constituent of the fraction. Moreover, attempts to isolate from the oxidation products (i) saccharic acid as potassium acid saccharate; and (ii) mannosaccharic acid as the diamide, were alike unsuccessful, and appeared to indicate the absence of both glycuronic and mannuronic acids from the original substance.

The precipitation of mucic acid is hindered, as is well known, by the presence of relatively large amounts of other organic matter, and as the fraction contains only 14 % of uronic anhydride it was thought possible that the small quantity of mucic acid had failed to come down. In further experiments, therefore, some of the sugars were removed by hydrolysis, and on neutralisation with barium hydroxide, the filtrate contained a barium salt which was precipitated by alcohol. 3 g. of this salt were submitted to bromine oxidation and yielded 0·32 g. of mucic acid, melting at 220°. The original presence of galacturonic acid was therefore inferred, and the above result confirmed by repetition.

The filtrate after separation of the mucic acid was examined for additional oxidation products and was evaporated to a syrup *in vacuo* at room temperature. Barium bromide was removed as it crystallised out and, after addition of 50 % potassium hydroxide, large amounts of potassium bromide were precipitated and filtered off. To the final clear filtrate a slight excess of glacial acetic acid was added, and after standing for three days, a fine crystalline precipitate was filtered off. On recrystallisation the product appeared as thick needle-shaped crystals with trapezoidal planes, identical with authentic crystals of acid potassium saccharate. Analysis of the crystals gave the following results:

0·0173 g. crystals yielded 0·0060 g. potassium sulphate, corresponding to 15·5 % potassium. Calculated: potassium 15·75 %.

The presence of glycuronic acid, in addition to galacturonic acid in Fraction III is therefore to be inferred.

No further sugars or sugar-acids were detected as constituents of this fraction, which as prepared is probably a sodium salt. The parent substance is composed of polysaccharides which yield on hydrolysis arabinose, galactose, galacturonic acid and glycuronic acid, and contain methoxyl groups. As in the case of Fraction I, there is no evidence that the product is a chemical entity; indeed it is most probable that both fractions are complex mixtures.

Isolation of barium salts of aldobionic acids.

It has been shown that hydrolysis of the mucilage for 2 hours with 4 % sulphuric acid suffices to precipitate the cellulose. In the experiment to be described, the hydrolysis, after separation of the cellulose, was continued for a further 16 hours, at the end of which period the solution was neutralised, while still warm, with barium carbonate. The precipitated sulphate was filtered off, the filtrate evaporated to small bulk under diminished pressure and poured into 95 % alcohol. The flocculum was separated by decantation and filtration, and after twice redissolving and reprecipitating was finally obtained as a white powder on drying in the usual manner.

The yield of salt from 50 g. of mucilage was only 0·85 g. so that an extensive examination was precluded. The following analytical results were obtained:

	%
Barium	21·70
On an ash-free basis:	
Uronic anhydride	51·00
Methylpentose	17·4
Hexose (by difference)	31·6
Furfuraldehyde	9·59
Furfuraldehyde from uronic anhydride (calc.)	8·50
Residual furfuraldehyde	1·09

The residual furfuraldehyde would correspond to less than 2 % of pentosan and may be neglected. For an aldobionic acid mixture of hexose-uronic acid and methylpentose-uronic acid in the proportions of 2: 1, the calculated analytical results are as follows:

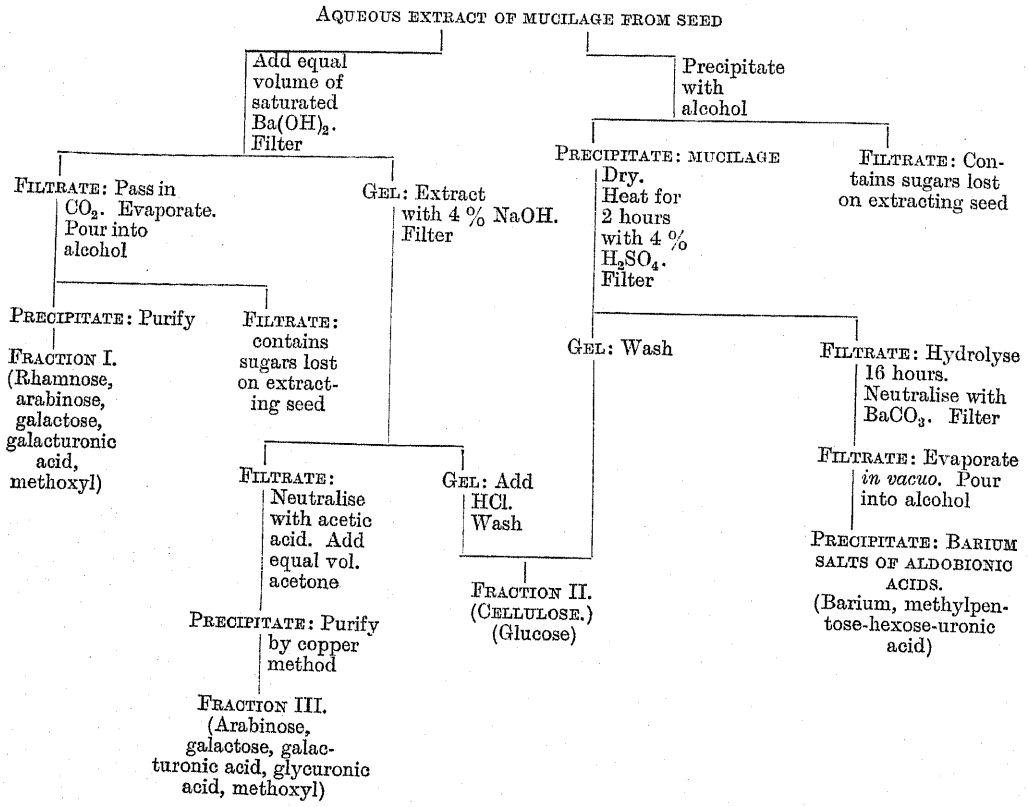
	%
Uronic anhydride	50·3
Methylpentose	16·1
Hexose	33·6

Having regard to the errors of the analytical methods involved, this is a sufficiently close approximation to the experimental figures above.

The product was obtained after prolonged hydrolysis under conditions which would destroy free uronic acids, and the results indicate that relatively stable aldobionic acid nuclei exist in the mucilage.

The operations described in the experimental section and the nature of the products obtained are indicated in the following scheme.

Scheme showing separation of mucilage into three fractions.



DISCUSSION.

From the foregoing it appears that the mucilage of mustard seed may be regarded as a type of compound cellulose in which the non-cellulosic moiety is composed of acid polysaccharides. The appearance of cellulose in the mucilage to the extent of roughly 50 % was unexpected, since there seems to be no reference in the literature to such a possibility. That there are other cases of its occurrence is indicated by experiments now proceeding on the mucilage of linseed, in which there appears to be a small proportion of cellulose, which has possibly been overlooked by other workers.

In the light of recent results by American workers, the existence in the mucilage of aldobionic acids was to be expected and it seems probable that a rhamnose-galacturonic acid and possibly a galactose-galacturonic acid are present. Challinor, Haworth and Hirst [1931], in a study of the galactose-glycuronic acid of gum arabic, have shown that the galactose residue is joined in the 6-position with the reducing group of the uronic acid residue. Such questions of linkage are outside the scope of the present communication, but it may be remarked in passing that in the case of rhamnose-galacturonic acid

neither the 5- nor the 6-position appears to be available for the purpose of linkage with the uronic acid residue.

The solubility relations of the complex polysaccharides constitute a physico-chemical problem of considerable interest. In the present instance cellulose, symbol of stability, completely insoluble in water, is rendered readily soluble by the presence of the acid polysaccharide moiety. The stability of the solution has been seen to be speedily affected by the addition of alkali or by warm dilute acid; the change, however, is not solely a physical one, but appears to involve some chemical rupture. On examination of the various products described in this paper, a possible correlation between solubility and the presence of uronic acids, or more particularly, carboxyl groups, suggests itself. Thus (i) cellulose, uronic anhydride content 3·4 %, is insoluble in water; (ii) Fraction III, uronic anhydride content 14·8 %, is soluble only with difficulty and gives a dense opaque solution; (iii) Fraction I, uronic anhydride content 26·7 % on a barium-free basis, is easily soluble in cold water.

The temptation to ascribe increasing solubility to increasing proportions of acid groups is considerable, but it is also probable that purely physical factors are concerned. In this connection it may be recalled that the solubilities of members of the pectin group are still difficult to explain; thus, pectic acid, with a uronic anhydride content of some 72 % is insoluble, whereas pectin in which the carboxyl groups are esterified, is comparatively readily soluble.

The probable mode of origin of uronic acids and pentoses from hexose material of the plant needs no prolonged discussion at this stage. The uronic acids may be produced by oxidation of the hexoses and in their turn suffer loss of carbon dioxide to yield pentoses [Haworth, 1929; Norman, 1929, 1; Norman and Norris, 1930]. The occurrence of the various hydrolysis products of the acid polysaccharides of the mucilage may be explained on these lines. Indeed, the mucilage itself seems to be an excellent example of an intermediate between cellulose, with all its hexosan units intact, and a typical pentosan, wherein the hexosan units of the original substance have suffered complete chemical change. In this light, the mucilage might be regarded as representing a half-way stage in the degradation.

Mild oxidation of pectin *in vitro* by means of hydrogen peroxide in the presence of a catalyst was shown by Norman and Norris [1930] to involve the production of substances of the nature of acid polysaccharides. The observation that a peroxidase system exists in cells closely associated with the mucilage-secreting cells of the mustard seed may indicate that oxidative processes do actually take place *in vivo* and may well account for the production of substances containing uronic acids.

The presence of a rhamnose-uronic acid in the mucilage constitutes a difficulty as its production from original hexose residues involves both oxidation and reduction; oxidation of a terminal CH_2OH group to produce the uronic acid, and reduction similarly to produce rhamnose. There is no experimental evidence which supports such a possibility, but it is conceivable that a

complete oxidation-reduction system may exist in the living organism which would be capable of effecting the complete change.

Whilst positive results have been recorded with reference to the composition and nature of the mucilage, the object of the present discussion has been largely to indicate the problems which still await solution.

Summary.

1. The mucilage of mustard seed is shown to consist of a complex of cellulose and acid polysaccharides.

2. The cellulose may be readily separated by warming with dilute sulphuric acid.

3. By addition of barium hydroxide solution to the mucilage solution a gel and also a soluble fraction composed of rhamnose, arabinose, galactose and galacturonic acid are obtained.

4. The gel obtained above may be further separated by the action of 4 % sodium hydroxide into cellulose and a fraction composed of arabinose, galactose, galacturonic acid and glycuronic acid.

5. The acid polysaccharides contain methoxyl groups in ether-linkage.

6. Aldobionic acids, probably rhamnose- and galactose-galacturonic acids are present in the mucilage.

7. A peroxidase system is present in cells situated close to the mucilage-secreting cells and may be responsible for the production of uronic acid residues from pre-existing hexosan material.

One of the authors (K. B.) is indebted to the Department of Scientific and Industrial Research for a grant received during the progress of the research.

Addendum.

While the above communication was in the Press, the existence of cellulose as a constituent of quince seed mucilage was reported by Cretcher and Renfrew [1932, *J. Biol. Chem.* **97**, 503].

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CXCIII. THE NON-SPECIFICITY OF THE PHENOLINDOPHENOL REDUCING CAPACITY OF LEMON JUICE AND ITS FRACTIONS AS A MEASURE OF THEIR ANTISCORBUTIC ACTIVITY.

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Early attempts at the fractionation of the antiscorbutic factor in lemon juice [Zilva, 1924, 1, 2] have revealed that active fractions possessed the property of reducing ammoniacal silver nitrate in the cold and of decolorising potassium permanganate. This fact taken in conjunction with the observations made on the inactivation of the vitamin by exposure to oxygen [Zilva, 1921; 1922; 1923] led the writer to pursue the inquiry with the object of ascertaining whether the reducing capacity of antiscorbutic concentrates was directly due to the vitamin itself or to substances, protective or otherwise, associated with it. It was found that the capacity for reducing ammoniacal silver nitrate or potassium permanganate in the cold, although closely associated with the antiscorbutic factor, was destroyed at a different rate from it [Connell and Zilva, 1924]. Efforts to modify active solutions in order to destroy their capacity for reducing ammoniacal silver nitrate and at the same time to leave the antiscorbutic activity unimpaired failed. A different technique of measuring the reducing capacity of the various fractions obtained from lemon juice, namely, that of titrating them with phenolindophenol (1-dimethylaminophenylindophenol) which was found to be decolorised by some of them was, therefore, eventually adopted. In this way the reducing capacity of various fractions could be determined with a good degree of accuracy. By using this procedure two main facts were ascertained by the writer [1927], (*a*) that it was possible to obtain fractions possessing little antiscorbutic activity, namely those precipitated by normal lead acetate from decitrated lemon juice at p_H 5·4, which possessed a reducing capacity equal to or sometimes even higher than that of the more active fractions obtained by precipitation with the same reagent from the same source at p_H 5·4–7·2; (*b*) that by adding to decitrated lemon juice phenolindophenol until it was no longer reduced the antiscorbutic activity was not destroyed, when tested immediately. In view of these observations the writer advanced a hypothesis that the

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substance (or substances) reducing phenolindophenol acted protectively in relation to the antiscorbutic factor in virtue of its reducing potential and it was referred to by him for the sake of convenience in all subsequent communications as "the reducing principle or factor." Later work indicated the possibility that certain reducing substances other than those reducing phenolindophenol and capable of having a deleterious effect on the antiscorbutic activity were present in some preparations [Zilva, 1930; Johnson and Zilva, 1932].

Recently Tillmans, Hirsch and collaborators [Trillhose, 1930; Tillmans, Hirsch and Hirsch, 1932; Tillmans, Hirsch and Siebert, 1932; Tillmans, Hirsch and Jackisch, 1932, 1, 2; Tillmans, Hirsch and Dick, 1932; Tillmans and Hirsch, 1932] produced evidence which in their opinion justified the assumption that the antiscorbutic factor itself was responsible for the reduction of phenolindophenol (2 : 6-dichlorophenolindophenol). The following is their evidence.

A fraction precipitated from decitrated lemon juice with normal lead acetate at p_H 5·4 (an equivalent of 1 cc. of the original juice reducing about 1·5 cc. of 0·001 *M* 2:6-dichlorophenolindophenol) neither prevented nor delayed death from scurvy of four guinea-pigs on a scorbutic diet, receiving a dose equivalent to 1 cc. per day, but protected another set of four guinea-pigs for 42 days when five times the dose was administered daily (the reducing capacity of the fivefold dose was 7·5 cc. 0·001 *M* 2:6-dichlorophenolindophenol). The fraction from the same juice which is precipitated between p_H 5·4–7·2 was not examined by them either for its reducing capacity or its antiscorbutic content. These writers consider, however, that evidence in support of the view that the vitamin itself is responsible for the reduction of the indicator is afforded by the fact that the reducing capacity of the fivefold dose of the fraction which is precipitated at p_H 5·4 is about equal to that of the protective dose of 1 cc. of decitrated lemon juice from which it is prepared, *i.e.* about 8 cc. of 0·001 *M* 2:6-dichlorophenolindophenol.

It is not at all surprising that by raising the dose of the fraction which is precipitated at p_H 5·4 some antiscorbutic protection was obtained. The separation of the vitamin by precipitation with normal lead acetate at various p_H values is by no means clear cut and a certain amount of the active principle is usually present in the fraction which is precipitated at p_H 5·4; sometimes this activity may be rather high [Zilva, 1929] . Had Tillmans and his collaborators tested the fraction obtained between p_H 5·4 and 7·2 prepared at the same time they would have found that the ratio of the antiscorbutic activity of this fraction to its reducing activity was decidedly higher than that found in the fraction precipitated at p_H 5·4. This is illustrated by Table I which gives the results of such a fractionation experiment performed by the writer in which the greater part of the antiscorbutic activity was contained in the p_H 5·4–7·2 fraction. These figures leave no doubt as to the lack of parallelism between the antiscorbutic activity and the reducing capacity for phenolindophenol.

Table I.

	Daily dose equivalent to original juice cc.	Reduction value of daily dose in cc. of 0·02 % phenolindophenol	Corresponding degree of protection from scurvy*. Av. score obtained from 3 animals for each test
Fraction precipitated at p_H 5·4	1·5	3	1
	3	6	2
	5	10	3
Fraction precipitated at p_H 5·4–7·2	1·5	2·6	5
	3	5·2	6
	5	8·7	7

* For an explanation of these numbers, see Table II.

Tillmans and his collaborators also claim that in the course of other investigations they found a consistent proportionality between the phenolindophenol-reducing capacity of a number of products and their antiscorbutic activity. In this connection about 114 tests comprising about 350 guinea-pigs, carried out by the writer over a period of 5 years on decitrated lemon juice and various fractions prepared from it, have been analysed by him for their capacity of reducing phenolindophenol and for their antiscorbutic potency. These tests were not specially selected but were chosen at random. Just as the biological test comprises in this case the composite effect of 60 days' dosing, so the phenolindophenol reducing capacity is the average of the daily titration values obtained for the same doses just before administration to the experimental animals. Each score in the biological tests comprises the average of three and sometimes even more guinea-pigs. As is well known, young healthy growing guinea-pigs of approximately the same weight vary very little in their response, as regards growth and development of scurvy, to the antiscorbutic factor, and consequently within the quantitative limits of the biological method the results obtained even in one test are incontrovertible. It is thus impossible to attribute a positive or negative result in the following experiments to anything but the presence or absence, as the case may be, of the antiscorbutic factor in the particular dose. The analysis of the tests is set out in Table II.

It will be seen that in five tests involving about 15 guinea-pigs, which received a daily dose with a reducing capacity of 9–10 cc. of phenolindophenol, little or no antiscorbutic protection was obtained. On the other hand a fair degree of antiscorbutic protection was obtained in three tests comprising about 9 guinea-pigs with a daily dose of a reducing capacity of only 2–3 cc. of phenolindophenol. The other doses also do not show a consistent relationship between antiscorbutic activity and reducing capacity. It will be further noted that the number of doses possessing some activity becomes greater and the doses show on the whole a higher activity when the reducing capacity reaches the figure of 4 cc. or thereabouts. This is most probably due to the fact that in any of these doses in which the vitamin was originally present, it was protected from inactivation during manipulation owing to the suitable

Table II. *The relationship of the phenolindophenol-reducing capacity of various preparations from lemon juice and their antiscorbutic activity as tested on guinea-pigs.*

Reduction value of daily dose in cc. of 0·02 % phenolindophenol	1–2	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10
Corresponding degree of protection from scurvy. Average score obtained from three animals for each test	0 0 0 0 0 0 0 0 0 0 0 2 2 . . .	0 0 0 0 0 0 0 0 1 2 2 2 5 5 6 .	0 0 0 0 0 0 0 1 1 1 2 2 3 3 5 6 7 . . .	0 0 0 0 0 1 2 2 4 5 6 7	0 0 0 0 1 1 2 3 5 5 6 8	0 0 1 1 2 3 3 5 5 6 6 6 8 . . .	0 0 2 2 4 6 8	1 5 6 7 8	0 0 1 1 1 5 5 5 5 7 7 8 . . . .

0 means death from scurvy within 30–35 days preceded by manifestation of scorbutic symptoms after 18–21 days and subsequent decline.
1 „ the same as 0 but with death ensuing after 35–40 days. It is doubtful whether the survival for these few extra days signifies the presence of any vitamin.
2 „ death from scurvy after 40–50 days.
3 „ death from scurvy after 50–60 days.
4 „ survival of guinea-pigs for 60 days. No growth. Severe scurvy at autopsy on chloroforming animals.
5 „ survival of guinea-pigs for 60 days. Initial growth but subsequent decline. Severe scurvy at autopsy on chloroforming animals.
6 „ survival of guinea-pigs for 60 days. Some growth. Slight scurvy at autopsy on chloroforming animals.
7 „ survival of guinea-pigs for 60 days. Moderate growth. Very slight scurvy at autopsy on chloroforming animals.
8 „ survival of guinea-pigs for 60 days. Good growth. No signs of scurvy at autopsy on chloroforming animals.

reducing potential of the medium. This could not be expected to happen when the reducing value was low, which would explain why in only 2 out of 17 tests a trace of antiscorbutic activity was detected with doses having a reducing capacity of only 1–2 cc.

Tillmans and his colleagues further maintain that the antiscorbutic activity which was observed by the writer in solutions which had been oxidised with phenolindophenol and had then been administered to the experimental animals immediately after oxidation was due to a reversible oxidation of the vitamin by the dye. This thesis will be dealt with in another communication.

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CXCIV. NARCOTINE AS THE ALLEGED PRECURSOR OF VITAMIN C.

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According to Rygh and Rygh [1931] and Rygh, Rygh and Laland [1932], when the neutralised juice from ripe oranges is extracted with peroxide-free ether, an antiscorbutic fraction, consisting of an oily substance with some crystals, is obtained. If on the other hand the juice from unripe green oranges is used the resulting product consists almost exclusively of crystalline narcotine, which does not possess any antiscorbutic activity. They therefore consider that narcotine is present in unripe oranges as a provitamin and as the fruit ripens[2] it is converted into vitamin C. This view received further support from an observation made by them [Rygh and Rygh, 1931; 1932] that when methyl*nor*narcotine, as prepared by demethylation of narcotine, is administered to guinea-pigs on a scorbutic diet, the development of the usual scorbutic lesions is prevented, although the death of the animals supervenes within about 5 weeks. The above experiments were repeated by us but we were unable to confirm any of the results obtained by Rygh and his collaborators.

Attempted isolation of narcotine from orange juice.

The freshly expressed juice (2000 cc.) of 214 green, unripe oranges (Valencia Late) from South Africa was extracted with purified ether (twice). After being made slightly alkaline to litmus the juice was again extracted with purified ether (four times). These four ether extracts were united, washed with water and dried with anhydrous sodium sulphate. The residue left after removal of the ether was examined for the presence of narcotine. No fraction with the characteristic properties of narcotine could be isolated but a slight precipitate was produced with Mayer's reagent. The precipitate did not give Labat's colour reaction [Labat, 1909] nor did it give any narcotine on decomposition with hydrogen sulphide. Narcotine when precipitated with Mayer's reagent

[1] Member of the Scientific Staff of the Medical Research Council working with grants for assistance and expenses.

[2] There is no evidence that unripe fruits or vegetables have a lower vitamin C concentration in their juices than ripe ones.

gives this reaction and when the precipitate from as little as 0·3 mg. of narcotine is decomposed with hydrogen sulphide the presence of the alkaloid can be detected.

In view of the weakly basic properties of narcotine the above first ether extracts were also examined for the presence of this alkaloid but no narcotine could be found.

Three other varieties of orange: South African Navel (unripe), Seville (partially unripe) and Jaffa (partially unripe) were examined in the same way with similar results. In all these experiments the efficiency of the extraction process was checked by adding narcotine to a portion of the orange juice and extracting as indicated above. In each case the narcotine was readily recovered as crystals which melted at 173° and after recrystallisation at 176°.

Ott and Packendorff [1932] have reported the isolation of narcotine from lemons. In one case a yield of 600 mg. of the crude product was obtained from 600 lemons. In other experiments the yield was much less and at times no narcotine could be obtained.

Attempted preparation of antiscorbutic concentrate from lemon juice by Rygh, Rygh and Laland's method.

Lemon juice was concentrated to about one-fifth of its volume at 25° under reduced pressure and the concentrated solution extracted with peroxide-free ether in a mechanical shaker in an atmosphere of pure nitrogen until no more colouring matter was removed from the lemon juice (three extractions). It was then neutralised to litmus using 40 % NaOH at first and finally 10 % NaOH and extracted again as above three times with peroxide-free ether. The combined second ethereal extracts were dried over $CaCl_2$ and concentrated at 30° under reduced pressure to a small volume to which 1 % aqueous tartaric acid solution was added. The residual ether was then distilled off also under reduced pressure. As the ether was removed some solid matter separated out. We found that when the ethereal extract was taken to dryness it was difficult to dissolve the entire residue in tartaric acid. In the biological tests an evenly distributed suspension of the above preparation was therefore used. The doses were prepared daily and were administered immediately, avoiding exposure to air as much as possible. During the week-end a double dose was given. Doses of $\gamma/10$, 1γ, 1 mg. and 2 mg. were tested, using three guinea-pigs (of about 300 g. wt.) on each dose. All the twelve animals succumbed to scurvy with the usual symptoms as if they were on the basal diet alone, showing the characteristic picture of the disease at the *post mortem* examination.

An examination of methyl*nor*narcotine for antiscorbutic activity.

We have already reported [Smith and Zilva, 1932] some negative results obtained with narcotine which had been hydrolysed with concentrated hydrochloric acid continuously for 48 hours and 72 hours respectively. Several more preparations demethylated under various conditions have since been

tested and were also found to be inactive. Demethylation of narcotine as carried out by Matthiessen and Foster [1867; 1868] and by Matthiessen [1869, 1, 2] yielded in our hands very impure methyl*nor*narcotine and dimethyl-*nor*narcotine containing much oxidised material. When demethylation was carried out in an atmosphere of nitrogen the rate of the reaction was found to vary in accordance with the purity of the nitrogen; the presence of even traces of oxygen increases the velocity of the reaction. This will be seen from Table I which sets out the degree of hydrolysis obtained by heating 5 g. of narcotine with 50 cc. of concentrated hydrochloric acid at 100°.

Table I.

Conditions	% MeO in crude product	Time of hydrolysis, hours
Aerobic...	2·1	89
In commercial nitrogen	2·9	,,
Solution covered with half an inch of paraffin wax	3·6	,,
In purified nitrogen	6·4	,,
In highly purified nitrogen	10·2	,,

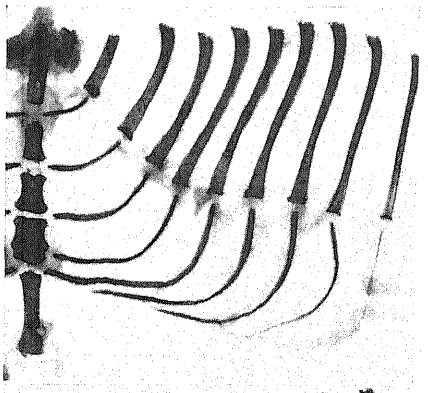
Methyl*nor*narcotine hydrochloride contains MeO, 7·4 %.
Dimethyl*nor*narcotine hydrochloride contains MeO, 14·2 %.

In this communication we are describing only two typical experiments, one dealing with methyl*nor*narcotine prepared under strictly anaerobic conditions and another in which less strict anaerobic conditions were observed.

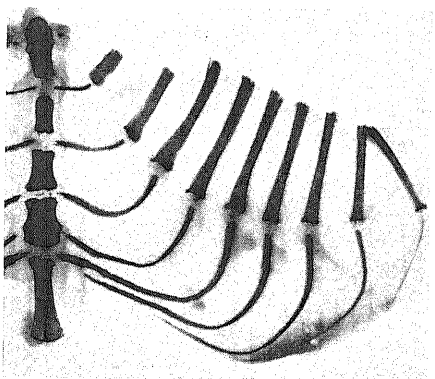
Exp. 1. 10 g. of narcotine in 100 cc. of freshly distilled oxygen-free hydrochloric acid were heated at 100° for 126 hours continuously under strict anaerobic conditions, using carbon dioxide as inert gas. The colourless reaction mixture became coloured rose-pink after being heated for 48 hours; after 72 hours a little methyl*nor*narcotine was deposited and increased in quantity as the reaction proceeded. On completion, the hydrochloric acid was distilled off *in vacuo* and the rose-pink amorphous residue was dissolved in 188 cc. of oxygen-free water, forming a light red-wine coloured solution (solid content, 0·0544 g. per cc.). All operations were carried out under strict anaerobic conditions.

Part of the solution was transferred to 1 cc. ampoules in an atmosphere of carbon dioxide and sealed without the solution coming into contact with air. The ampoules were reserved for biological tests, which are described below, and the remaining solution was used for the chemical examination of the product.

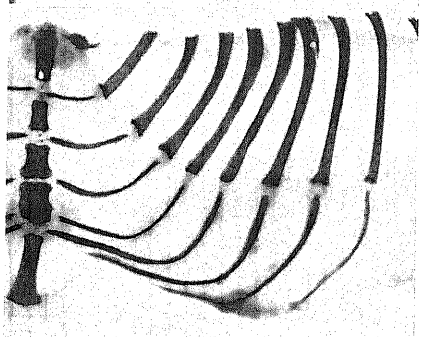
Chemical examination. The remainder of the solution was treated with sufficient concentrated hydrochloric acid to produce maximum precipitation. The white amorphous precipitate (6·0 g.) was filtered off as quickly as possible and dried over phosphorus pentoxide in a high vacuum. The precipitate developed a slight grey-lavender colour on drying. Found, after drying in a vacuum at 95° for half an hour and cooling in an atmosphere of dry nitrogen; C, 56·7; H, 5·2; N, 3·2; Cl, 8·5; MeO, 7·5 %. Calculated for $C_{20}H_{19}O_7N$, HCl;



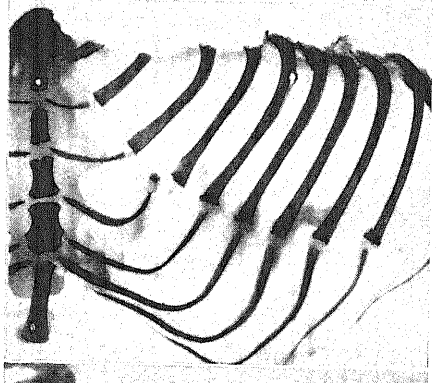
10 γ



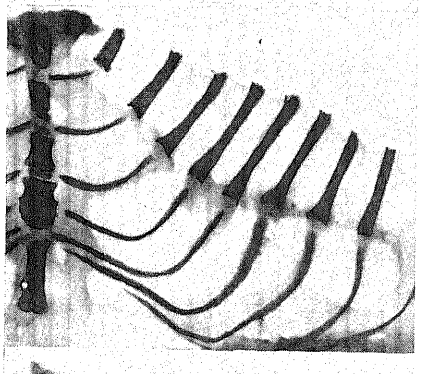
20 γ



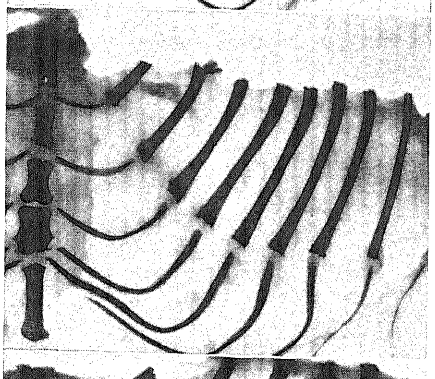
30 γ



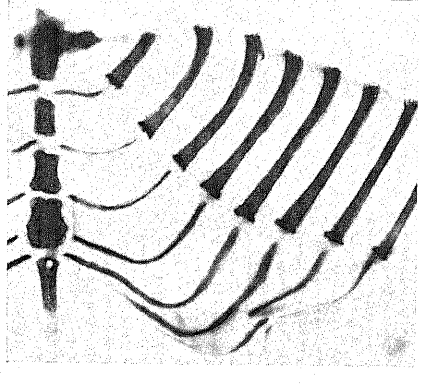
100 γ



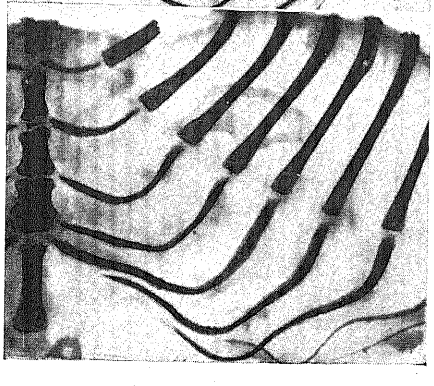
1·4 mg.



2·7 mg.



Negative control



Positive control

C, 56·9; H, 4·8; N, 3·3; Cl, 8·4; MeO, 7·4 %. The mother-liquor was water-clear and contained nothing except a trace of methyl*nor*narcotine.

Fractionation of the remaining methyl*nor*narcotine (5·6 g.) gave 0·5 g. of less soluble material (MeO, 6·5 %) which consisted of methyl*nor*narcotine hydrochloride, *nor*narcotine hydrochloride and oxidised material; 0·3 g. of more soluble material (MeO, 8·8 %) which consisted of a mixture of methyl-*nor*narcotine hydrochloride and dimethyl*nor*narcotine hydrochloride; and 4·1 g. of methyl*nor*narcotine hydrochloride (MeO, 7·4 %). The latter still contained a trace of oxidised material which was removed by prolonged treatment with acetone in the cold.

Methyl*nor*narcotine hydrochloride is a colourless, amorphous powder, M.P. 253° (corr. dec.), $[\alpha]^{22°}_{\text{Hg yellow}}$ + 333°, $[\alpha]^{22°}_{5461}$ + 382° (*c*, 1·2 in water). It is readily soluble in water, almost insoluble in dilute hydrochloric acid, sparingly soluble in concentrated hydrochloric acid. It does not crystallise as stated by Rygh but separates from solvents in small white granules. The base is conveniently prepared by dissolving the hydrochloride in pyridine and pouring the solution into water. Methyl*nor*narcotine is a cream-coloured amorphous powder, melting point 227° (corr. dec.). It is sparingly soluble in most organic solvents but dissolves readily in pyridine. Attempts to prepare crystalline salts gave amorphous products which showed no tendency to crystallise. The esters prepared by acetylation or benzoylation were also amorphous. The base tends to oxidise on drying for analysis; found C, 61·7; H, 5·1; N, 3·6; MeO, 7·9 %. $C_{20}H_{19}O_7N$ requires C, 62·3; H, 5·0; N, 3·6; MeO, 8·0 %.

Biological test. The following doses were tested, 10γ, 20γ, 30γ, 100γ, 1·4 mg. and 2·7 mg., using 6 guinea-pigs (of about 300 g. wt.) on each dose. A layer of liquid paraffin was introduced immediately after opening the ampoules in order to protect the contents from the air. A quantity of the solution equivalent to the dose was then quickly removed by means of a graduated capillary pipette and diluted with oxygen-free, distilled water. The time which elapsed between the removal of the dose from the ampoule and the end of the delivery of the dose was under a minute. All the animals developed scurvy in the usual time and died of the disease after about 30–35 days. The *post mortem* examinations revealed typical scurvy. X-ray photographs were taken of the ribs of all the animals in this experiment but no differences between the animals which received methyl*nor*narcotine and those which were on a basal diet alone, such as were observed by Rygh and Rygh [1931], could be discerned. This will be seen from Plate VI in which X-ray photographs of the ribs of one animal in each group are given. We should like to take this opportunity of expressing our indebtedness to Miss H. M. Bruce of the National Institute for Medical Research, Hampstead, for kindly preparing the radiographs.

Exp. 2. Demethylation was carried out under less strict anaerobic conditions. 10 g. of narcotine in 100 cc. of concentrated hydrochloric acid were heated at 100° for 50 hours continuously in an atmosphere of commercial

nitrogen. The hydrochloric acid was then distilled off and the residue (10·2 g.) dissolved in oxygen-free water (112 cc.). Part of the solution (50 cc.) was sealed up in 1 cc. glass ampoules under commercial nitrogen for biological tests. The crude methyl*nor*narcotine (MeO, 8·7 %) was found to consist of about 80 % methyl*nor*narcotine with 20 % of dimethyl*nor*narcotine and a trace of oxidised material.

The tests in this experiment were carried out under the same conditions as in Exp. 1 except that four animals only were used on each dose. As in Exp. 1 no deviation in the behaviour of the experimental animals from that of those on a scorbutic diet alone could be observed.

Since the completion of this investigation the results of Dalmer and Moll's [1932] repetition of Rygh's work have come to our notice. Like ourselves they were unable to isolate narcotine from oranges or to prepare active concentrates from lemons by Rygh, Rygh and Laland's method, nor could they detect any antiscorbutic activity in methyl*nor*narcotine. Tillmans and Hirsch [1932] and Harris *et al.* [1932] also could not detect any antiscorbutic activity in methyl-*nor*narcotine.

Summary.

Narcotine could not be detected in unripe or partially ripe oranges.

Attempts to prepare an antiscorbutic concentrate from lemon juice according to Rygh, Rygh and Laland's method failed.

The administration of a number of preparations of methyl*nor*narcotine, obtained by demethylation of narcotine under various conditions, to guinea-pigs on a scorbutic diet failed to modify the development or character of the ensuing scorbutic symptoms. The experimental animals died in the usual time of typical scurvy.

Our thanks are due to Mr A. Bennett and Mr H. C. Clarke for carrying out the analyses in this investigation.

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CXCV. FURTHER OBSERVATIONS ON THE DISTRIBUTION OF FAT AND GLYCOGEN AFTER THE SUBCUTANEOUS INJECTION OF EXTRACTS OF THE POSTERIOR PITUITARY GLAND.

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In a recent paper the effects on the blood-sugar, muscle- and liver-glycogen, and liver-fat, which ensued as the result of a single subcutaneous injection of either pitressin or pitocin, were described [Hynd and Rotter, 1932]. Pitressin, in its effects on the metabolism of both fat and carbohydrate, appeared to be much more potent than pitocin. Indeed, so indefinite were the results obtained with pitocin that it was doubtful whether any significance could be attached to the slight changes observed. Nevertheless, the results of preliminary experiments with pituitrin suggested that pitocin was not entirely without effect. Moreover, Raab [1930] concluded that both pitressin and pitocin exercise a certain influence on the phosphatides of the blood similar to that of pituitrin.

Accordingly, as it seemed likely that some light on the problem might accrue from a careful study of the effects produced by the injection of (*a*) pituitrin, that is the unseparated posterior pituitary extract, and of (*b*) artificial mixtures of pitressin (the pressor constituent) and pitocin (the oxytocic constituent), such an investigation was commenced and the results obtained are dealt with in the present communication.

Experimental.

As the effect of pituitary extracts on the distribution of glycogen and fat is less definite in the case of animals fed on a carbohydrate-free diet than in those receiving abundant carbohydrate [Hynd and Rotter, 1932], the present series of experiments was confined to albino rats fed on bread and milk. Each rat when used for experiment weighed approximately 100 g. A single dose of the hormone was administered subcutaneously at varying intervals before killing the animal by stunning. The effects on the blood-sugar, liver- and muscle-glycogen, and liver-fat were investigated, the same technique as previously described being followed. The sex of the rat was always noted, but contrary to expectations, no difference in the results obtained with male and female animals was detected. The pituitary extracts used were all provided

by Parke, Davis and Co., and as a rule contained chloretone as a preservative. It may be added at this point that through the kindness of Parke, Davis and Co., we were enabled to carry out a set of experiments with pitressin, specially prepared for research purposes and free from preservative. As the results obtained with this material were identical with those already reported [Hynd and Rotter, 1932] for pitressin containing chloretone, it seems reasonable to draw the conclusion that the presence of chloretone as a preservative does not interfere with the effects produced by the posterior pituitary hormones on the metabolism of fat or carbohydrate.

(*a*) *Effect of pituitrin.*

Blanching and other symptoms, similar to those described for rats injected with pitressin, were noted in animals receiving pituitrin. Time-curves after the subcutaneous injection of 2·5, 10 and 20 units of pituitrin were completed. In experiments with this extract 24 rats were used, and identical results were obtained with two distinct samples of the hormone. To economise space only the average values of the results obtained are summarised in Table I.

Table I. *Analyses of carbohydrate-fed rats after the subcutaneous injection of pituitrin.*

No. of units	Time after injection (hours)	Blood-sugar %	Muscle-glycogen %	Liver-glycogen %	Liver-fatty acids %	Iodine value of fatty acids
2·5	0	0·15	0·55	3·5	3·7	120–140
	1	0·165	0·78	3·34	3·44	127·5
	2½	0·18	0·66	2·31	4·3	—
	5	0·18	1·05	2·3	3·68	104
	7	0·148	1·1	2·43	3·5	110
	12	0·17	1·34	2·65	3·5	90·5
	14	0·15	1·58	2·65	—	109
	15	0·16	1·87	3·95	3·5	105
	16	0·11	1·05	4·16	3·6	106·5
	17½	0·16	1·59	8·8	3·1	101·5
	18	0·12	1·02	10·6	3·66	—
	24	0·14	1·03	7·95	2·0	—
	36	0·16	0·95	3·57	3·09	121
10	2½	0·187	0·94	2·88	—	74
	7	0·176	1·18	2·8	3·45	110
	17½	0·16	1·1	4·6	3·5	96
20	5	0·16	1·27	2·3	3·45	114
	7	0·12	1·11	1·95	4·05	119
	9	0·21	0·98	2·54	4·18	111
	15½	0·158	1·02	5·2	4·6	88

(*b*) *Effect of a mixture of pitocin and pitressin.*

As pituitrin contains 10 units of pitressin and 10 units of pitocin per cc., the mixture used for injection was prepared so as to contain an equal number of units of each constituent, the amount injected being then adjusted so as to contain 2·5 units of pitocin and 2·5 units of pitressin. Later it was found more convenient to inject the animal in one flank with the necessary amount of pitocin and in the other with the pitressin. No difference in the results obtained for the two methods of administering the extracts was detected.

Experiments have been carried out with the mixture of pitocin and pitressin in the case of 24 rats. The general symptoms produced were similar to those observed in rats injected with pitressin alone. The average values for the results of the chemical analyses are given in Table II.

Table II. *Analyses of carbohydrate-fed rats after the subcutaneous injection of a mixture of pitocin (2·5 units) and pitressin (2·5 units).*

Time after injection (hours)	Blood-sugar %	Muscle-glycogen %	Liver-glycogen %	Liver-fatty acids %	Iodine value of fatty acids
0	0·15	0·55	3·5	3·7	120–140
1	0·18	0·81	3·08	4·1	129·5
2½	0·18	1·46	2·45	3·69	116·5
3½	0·13	0·63	2·05	3·83	113
5	0·18	1·56	2·55	3·40	115
6	0·15	1·26	2·2	3·60	127
7	0·187	1·23	2·65	3·1	104
15	0·16	1·89	5·4	3·51	127
18	0·17	0·64	3·65	4·33	130
19	0·15	1·45	3·67	3·48	140
21	0·16	1·85	4·1	4·17	124
24	0·13	0·76	1·23	2·55	108

Discussion.

As the several functions affected by extracts of the posterior pituitary are so diverse, it is probable that each function is influenced by a different hormone. But so far no single hormone of the posterior pituitary gland has been isolated in a pure condition [Schäfer, 1931]. Nevertheless Kamm *et al.* [1928] separated pituitrin into two fractions, pitocin and pitressin, the former containing the oxytocic principle, while the latter is responsible for the vaso-pressor effect. The same workers [1931] claim that there is as yet no evidence that more than two hormones are present in pituitrin, and that on recombining the two separated hormones in the proportion of one oxytocic to one pressor unit, an extract with all the activities of the original pituitrin is reproduced. Hence the mixture of pitressin and pitocin used in the experiments recorded above should produce changes identical with those resulting from an equivalent dose of pituitrin. Though this is doubtless the case with regard to the effect on blood pressure and the contraction of uterine muscle (the two methods usually adopted for assay), comparison of the results summarised in Table I with those in Table II shows that this identity does not apply to the changes in glycogen and fat distribution.

The effect of a mixture containing pitocin and pitressin in the proportions in which they are alleged by Kamm to occur in pituitrin is very similar to that already reported to result from an injection of pitocin alone. As in the case of the latter hormone, the only definite change is an increase in muscle-glycogen. This increase occurs almost immediately, values varying from 1 to 2 % being observed 2½ hours after the injection, and the high level is maintained for at least 24 hours. There is also a possible slight increase in blood-sugar, not noticed after pitocin alone, but no infiltration of fat into the liver

and no change in the iodine value of the liver-fat occur. There is a tendency for the liver-glycogen to fall during the initial 5 hours, and then to increase somewhat over the normal. These changes however are not marked, and graphic representation of the results would emphasise the difference between the effects produced by what might be termed "artificial" pituitrin and those resulting from "natural" pituitrin.

Injection of pituitrin brings about changes in both liver- and muscle-glycogen similar to those which follow the injection of pitressin. During the first 5 hours the liver-glycogen falls to about 1 %. Thereafter there is a steady and well-marked increase, which reaches its maximum of over 9 % about the 17th hour after injection. Return to the normal value of 3·5 % is attained by the 36th hour. During these changes in liver-glycogen, the muscle-glycogen is maintained at an abnormally high level, namely about 1 % or twice the normal control value for rats under the same dietetic conditions. Consequently, though glycogen may be transferred from liver to muscles and *vice versa*, such a transference is altogether inadequate to account for all the changes observed in the distribution of the glycogen reserves. Moreover, attention has previously been drawn to the fact that the magnitude of the changes in glycogen distribution produced by pitressin is independent of the size of the dose employed. Similar results are obtained with pituitrin, which in this regard appears as efficient as pitressin.

On the other hand, pituitrin is much less effective than pitressin in altering the distribution of fat, and the effect produced varies somewhat with the dose employed. Thus after the injection of 2·5 units of pituitrin no infiltration of fat into the liver was detected, though a fall in iodine value (less than 120) made it evident that the character of the fat had changed to some extent. Even after 20 units of pituitrin fatty infiltration did not always occur. When it was obtained, it was never so marked as that after 2·5 units of pitressin. As Raab has reported that pituitrin caused a more marked decrease in the blood-fat level than pitressin, this finding is not what one would expect. Nevertheless it is in agreement with the observation of Coope and Chamberlain [1925]. These workers showed that while a comparatively small dose of pituitrin brought about an infiltration of fat into the liver of a rabbit, a large dose of at least 20 units was not always successful in effecting a like change in the liver of a rat. To reconcile Raab's finding that pituitrin has a more definite effect than pitressin in decreasing the blood-fat, with the results now reported that pitressin is more potent than pituitrin in increasing liver-fat, it is necessary to postulate that pitocin also plays a definite rôle in fat metabolism.

Even though pitocin acting alone was found to have little or no effect on the fat content of the liver, the presence of the oxytocic constituent in pituitrin evidently interferes with the accumulation of fat in the liver. It is remarkable that insulin, as shown previously by Coope [1925], has a like function. Though the same result is achieved by pitocin as by insulin, it is highly probable that these two hormones bring about this effect on the liver-fat by entirely

different processes. However, in the present state of knowledge it is impossible to say whether pitocin prevents the accumulation of saturated fat in the liver by inhibiting the actual transference of fat, or by facilitating the oxidation and utilisation of fat brought to the liver, thereby keeping the liver-fat approximately normal both in character and amount. Certain observations of Raab [1928] are opposed to the former view, and the alternative mechanism would generally be regarded as characteristic of the pancreatic hormone. However, there is considerable experimental evidence which shows the interdependence of the pituitary body and the islets of Langerhans, and clearly indicates that the injection of insulin stimulates the pituitary gland to increased secretion [Eaves, 1926; Geiling and Britton, 1927; La Barre, 1930, 1, 2]. Consequently it must not be overlooked that the facilitation of fat metabolism ascribed to insulin may not be due directly to insulin, but to a hormone (pitocin) secreted by the posterior lobe of the pituitary.

Further, such a view would be consistent with the fact recently established by Himwich and Haynes [1931] that pitocin brings about an increase in metabolism of 15 % on the average. These same workers have shown that pitressin, on the other hand, reduces the basal metabolic rate by 22 %. In pituitrin, therefore, there are present two hormones which are antagonistic with regard to their effect on the total metabolism. Elmer and Ptaszek [1930] have also demonstrated that pitressin and pitocin are antagonistic with regard to their action on intestinal peristalsis. The results now communicated show that while pitressin during the first 5–7 hours after injection causes an accumulation of liver-fat and between the 12th and 24th hours storage of liver-glycogen, pitocin entirely inhibits the former and depresses the latter change to a considerable extent. Many factors, such as dosage, method of administration of the hormone, the species of animal and the experimental conditions employed, contribute to the confusion which exists in the literature regarding the effect of posterior pituitary extracts on the metabolism of fat [Raab, 1930] and carbohydrate [Geiling, Campbell *et al.*, 1927]. The opposing actions of the two recognised constituents of pituitrin doubtless explain many of the inconsistencies reported, since the extracts employed by different observers may have contained varying amounts of the pressor and oxytocic principles.

Fukui's observation [1925] that posterior pituitary extracts produce no significant changes in the glycogen reserves seems to find support from recent work by Lawrence and McCance [1931] on the fasting rat, and by Bischoff and Long [1931, 1, 2] on rabbits injected with pitressin or pituitrin. Nevertheless, using somewhat different experimental conditions, it is evident from the results of the present experiments on rats that the posterior pituitary has an important influence on both carbohydrate and fat metabolism, but the mechanisms involved are much more complex than have been supposed.

Hitherto, in experimental studies of the effects of pituitrin, attention has been confined to the first few hours after administration of the hormone. It seems expedient therefore to emphasise that the experiments in the present

investigation have been continued until a return to the normal was obtained. Under the experimental conditions adopted this required from 24 to 36 hours. In this way, as already indicated, remarkable changes in liver- and muscle-glycogen have been detected, and, as these changes are at a maximum about the 15th–17th hour after injection, they have been missed by other observers.

During the initial stages, in addition to the infiltration of fat into the liver, a transference of glycogen from the liver to the muscles seems to be the effect produced. However, in the later stages when glycogen is being stored in the liver, a shift of glycogen from the muscles cannot explain the observed accumulation of glycogen in the liver, since the muscle-glycogen remains high. Evidently a synthesis from some outside source must have taken place, but it is not yet clear what this source is. The simultaneous changes in the fat and glycogen of the liver after the injection of pitressin provide an excellent example of Rosenfeld's law, and are suggestive of a conversion of fat into carbohydrate. However, the fact that in pituitrinised animals the glycogen changes are not necessarily accompanied by an infiltration of fat makes it doubtful whether such a conversion is involved.

The further question arises as to whether the effects occurring after a period of 15 hours should be attributed to the hormone actually injected, or whether they arise as secondary effects, produced by the compensatory secretion of some other hormone or hormones. Thus, La Barre [1930, 1, 2] holds that the hyperglycaemia which occurs after the administration of posterior pituitary extracts is followed by a hypoglycaemia, and he attributes the former to an increased excretion of adrenaline and the latter to hyperinsulinaemia. Many other observers, including Lambie [1926], Geiling and his co-workers [1927], and Bischoff and Long [1931, 1], have suggested that the effects of the posterior pituitary on carbohydrate metabolism involve the interaction of the suprarenals. The last-named authors, however, as a result of further experiments on adrenalectomised animals, have now changed their opinion [Bischoff and Long, 1931, 2]. Consequently until the exact mechanism by which the changes in question are produced is elucidated, it seems best to refer them to the posterior pituitary. Nevertheless the interplay of the endocrine secretions is certainly an important factor [Schäfer, 1931] but one regarding which speculation is better avoided.

Summary.

The subcutaneous injection of pituitrin into carbohydrate-fed rats produces effects on the liver-glycogen similar to those obtained with pitressin, namely a fall during the first 5 hours, followed by a rise, which is at a maximum about the 18th hour after injection.

The effect on the muscle-glycogen is also similar to that with pitressin, an increase during the first 5 hours to twice the normal, at which level it is maintained for at least 24 hours.

On the other hand, pituitrin is much less efficient than pitressin in producing an infiltration of fat into the liver.

An artificial mixture of equal parts by volume of pitressin and pitocin does not produce the same changes in liver-glycogen and fat as does the "natural" pituitrin, fatty infiltration being entirely inhibited and the increase in liver-glycogen greatly depressed.

From the results obtained it is concluded that pitocin is antagonistic to pitressin in its effects on the liver-fat and the liver-glycogen.

As the effects produced by the several extracts vary with the interval elapsing after the injection, it is important to take account of the time during which the extract is allowed to act.

It is suggested that failure to do so, as well as the hitherto unrecognised antagonism between pitocin and pitressin, may account for many of the conflicting reports in the literature regarding the effect of posterior pituitary extracts on carbohydrate and fat metabolism.

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CXCVI. CHANGES IN THE COMPOSITION OF COW'S BLOOD AT THE TIME OF CALVING AND A COMPARISON OF THE BLOOD OF THE CALF WITH THAT OF ITS DAM.

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Relatively little information is available in the literature as to changes which may occur in the composition of the blood of the cow about the time of calving or as to the relationship between the composition of the blood of the calf and of that of its dam. In a recent paper Hodgson, Riddell and Hughes [1932] state that the calf at birth has a high blood-sugar level, the average being 100 mg. per 100 cc. at 1–6 days old, and that this value falls steadily to about 55 mg. at 2 years, and similar results were obtained by Anderson, Gayley and Pratt [1930]. They give no comparison between individual calves and their dams. Schwarz and Metzler-Andelberg [1928] found no significant alteration in the sugar of cow's blood immediately after calving.

Bogert and Plass [1923] found that, in the human being, the serum-Ca of the newborn child was higher than that of the mother and usually exceeded the upper limit for normal adults, the values for the child and mother at birth being 10·9 and 9·1 mg. per 100 cc. respectively. They state that at the end of labour the maternal serum-Ca is below the normal level for non-pregnant women. Robinson and Huffman [1926] also report a higher figure for serum-Ca of the calf than of the dam and state that during the first week after calving the dam's serum-Ca rises whilst that of the calf falls. Green and Macaskill [1928] could find no significant difference between the calf and its dam with respect to serum-Ca. Wilson and Hart [1932] report a fall in the serum-Ca level of cows within 3 days of calving, stating it to be more usual in cows which have lactated before. According to Robinson and Huffman [1926] the inorganic P is lower in the dam's blood than in the calf's blood at birth, but rises to a maximum within 18 hours and then falls again, whilst that of the calf tends to rise throughout the first week. Green and Macaskill [1928], working with veld-bred cattle of low milk yield, found that the inorganic P of the blood of the calf at birth was appreciably higher than that of the mother and rose sharply in the first week, beginning to fall during the second month. Wilson and Hart [1932] note a fall in inorganic P at calving, but state that it is not so consistent as the fall in serum-Ca. Green and

Macaskill [1928] observed no significant difference between the blood-Cl of the cow and its calf, whereas Robinson and Huffman [1926] note a high blood-Cl in the cow at calving followed by a steady fall, and a lower blood-Cl level for the calf than for its dam.

In the course of work in progress here, designed to obtain normal figures for dairy cows at different stages in their life cycle and to determine the factors responsible for variations in the different values, advantage was taken of the calving period to obtain further information on (1) the changes occurring in the chemical composition of the blood of the cow, immediately prior to, at the time of, and subsequent to calving; (2) the relationship between the chemical composition of the blood of the calf and of that of its dam at calving and during the first week after calving.

Method.

The results recorded in this communication were obtained from the study of 12 normal calvings. All the cows were 6 years old and dropping their fifth calf, except Cow No. 20 which was 8 years old. They had all been on a steady diet and regime for 3 years. As far as possible, blood samples were drawn on successive days for 2–3 days before calving was expected, immediately after calving in all cases, and then daily for 6–7 days, all bleedings being made at a regular hour each day, except for the sample immediately prior to and as near to the time of calving as possible. When the calves were strong enough to stand bleeding, samples were taken at birth, and thereafter every second day for one week. Owing to the fact that some of the cows did not calve exactly on the expected date and did not always give clear enough indication of approaching labour, the data for the period prior to calving are not quite so complete as had been hoped for.

The blood samples were drawn from the jugular vein in the case of both cows and calves. The following analytical methods were adopted.

Blood-sugar. Hagedorn and Jensen's method [1920].

Serum-Ca. Kramer and Tisdall's method [1921; Tisdall, 1923].

Inorganic P. Briggs's modification [1922] of the Bell-Doisy method. The precipitation of the proteins for this and the blood-sugar was done in the byre immediately after the samples were drawn, all other work being done in the laboratory.

Chlorine. Whitehorn's method [1920–21] modified by filtering off the silver chloride precipitate through a dry filter and titrating an aliquot portion of the filtrate with thiocyanate, using a ferric indicator, prepared by boiling ferric alum with nitric acid, instead of the solid salt.

Non-protein-nitrogen (N.P.N.). Folin's method [1922].

The data for each individual cow and calf are shown in Table I. These figures have been averaged for the cows and the calves (omitting the calves from Cows Nos. 11, 12 and 16, from which samples could not be obtained at

Table I. *Composition of the blood of cows and their calves just before, at, and for the first week after calving.*

All values are stated in mg. per 100 cc.

Days before or after calving	Blood-sugar	Serum-Ca	Inorganic P	N.P.N.	Chlorine	Blood-sugar	Serum-Ca	Inorganic P	N.P.N.	Chlorine
	Cow No. 4.					Calf No. 4.				
2 before	75·88	11·45	3·63	30·78	334					
1 „	90·84	10·90	3·44	23·08	331					
Calving	88·20	10·95	3·33	23·90	313	133·96	14·30	6·37	24·39	312
1 after	77·64	9·45	3·55	33·15	335					
2 „	60·04	9·75	5·40	34·09	318	106·68	12·50	4·82	32·43	292
3 „	65·32	9·95	5·44	32·97	323					
4 „	56·52	10·00	6·03	24·19	332	95·24	12·40	5·97	35·08	286
5 „	67·08	10·45	5·58	25·44	333					
6 „	69·72	11·00	6·03	25·86	329					
	Cow No. 5.					Calf No. 5.				
2 before	60·04	11·05	4·41	27·65	336					
1 „	60·04	11·50	4·38	28·15	339					
Calving	70·60	9·15	2·25	29·85	328	117·24	13·70	5·58	40·27	284
1 after	70·60	9·15	2·55	27·53	330					
2 „	60·04	9·05	3·63	30·61	340	104·92	11·15	5·58	40·81	278
3 „	57·40	9·30	4·96	26·68	302					
4 „	67·08	8·90	5·15	32·36	301	83·80	11·40	4·96	29·41	291
5 „	50·36	9·05	5·49	27·77	313					
6 „	49·48	8·85	6·49	28·99	309	89·96	11·85	6·43	31·58	301
7 „	53·00	8·85	5·62	24·49	316					
	Cow No. 7.					Calf No. 7.				
1 before	59·16	11·05	2·52	24·00	335					
Calving	77·64	10·05	2·36	20·83	325	90·84	12·75	5·62	24·19	313
1 after	69·72	11·15	3·00	20·40	315					
2 „	60·04	10·45	4·62	26·66	328	100·32	11·90	5·58	25·97	306
3 „	59·16	11·70	5·97	24·00	327					
4 „	65·32	11·20	5·15	25·22	326	95·24	12·30	5·40	29·13	291
5 „	53·88	11·40	7·03	26·20	322					
6 „	60·04	10·90	5·36	25·00	331	92·60	12·40	5·82	28·99	306
	Cow No. 10.					Calf No. 10.				
2 before	76·76	11·00	4·47	38·22	324					
Calving	67·96	8·35	3·01	35·08	320	106·68	13·75	5·00	43·38	282
1 after	61·80	9·05	3·67	31·41	320					
2 „	79·40	10·10	5·58	30·30	314	124·78	12·50	4·19	35·08	265
3 „	67·96	10·10	5·58	31·41	316					
4 „	60·92	10·50	5·92	34·09	321	124·28	14·05	5·53	34·68	270
5 „	53·00	10·05	6·68	34·09	322					
6 „	69·72	10·20	6·13	36·58	322	97·88	12·15	5·92	35·29	293
	Cow No. 11.					Calf No. 11.				
2 before	72·36	11·55	6·31	24·89	335					
1 „	60·04	11·40	5·87	34·68	347					
Calving	90·84	8·65	3·75	28·02	330					
1 after	70·60	9·45	4·93	31·41	325					
2 „	68·84	9·50	4·85	33·15	325					
3 „	73·24	10·45	5·67	33·15	331	128·28	13·20	5·62	34·48	297
4 „	58·28	11·35	6·68	29·55	314					
5 „	66·20	—	7·10	29·13	313	132·20	—	6·55	39·73	295
6 „	78·52	11·25	6·37	27·15	324					
7 „	64·44	10·95	6·32	27·15	324					
	Cow No. 12.					Calf No. 12.				
2 before	52·12	11·30	4·78	30·30	344					
1 „	52·12	10·45	—	30·61	345					
Calving	69·72	9·75	3·67	27·15	334	152·44	—	6·37	—	—
1 after	64·44	10·30	8·42	25·75	345	125·16	11·90	4·50	34·48	302
2 „	72·36	8·65	7·84	25·97	331					
3 „	63·56	9·05	6·61	26·90	339	118·12	11·85	4·75	39·20	298
4 „	66·20	10·30	5·97	25·97	333					
5 „	—	—	—	—	—	114·60	11·90	4·85	35·39	293
6 „	72·36	10·95	4·82	25·75	325					

Table I (*cont.*).

Days before or after calving	Blood-sugar	Serum-Ca	Inorganic P	N.P.N.	Chlorine	Blood-sugar	Serum-Ca	Inorganic P	N.P.N.	Chlorine
	Cow No. 13.							Calf No. 13.		
1 before	67·08	10·75	4·02	25·54	338					
Calving	89·08	9·60	2·60	29·85	325	139·24	14·20	4·85	40·27	311
1 after	50·36	10·05	5·32	—	—					
2 „	70·60	10·25	5·15	34·09	312	130·44	12·55	5·58	29·55	299
3 „	62·68	9·85	4·93	32·26	318					
4 „	57·40	9·80	3·82	33·15	328	99·64	12·45	5·11	32·78	294
5 „	54·76	10·00	4·47	33·89	319					
6 „	56·52	10·45	3·55	33·89	345	94·36	12·00	6·49	32·97	288
7 „	56·52	10·50	4·32	29·85	324					
	Cow No. 14.							Calf No. 14.		
Calving	65·32	9·55	4·89	27·41	337	122·52	14·10	5·07	40·27	301
1 after	52·12	8·75	6·75	27·65	336					
2 „	56·52	8·00	5·53	26·90	340	119·88	13·90	5·58	33·51	290
3 „	71·48	9·70	5·82	29·95	333					
4 „	55·64	9·60	5·72	31·41	324	111·08	14·00	5·77	35·08	278
5 „	55·64	9·40	5·87	30·61	329					
6 „	60·92	9·45	5·77	29·70	328	117·24	13·15	5·92	40·27	299
7 „	62·68	9·45	6·03	33·89	327					
	Cow No. 15.							Calf No. 15.		
1 before	67·08	10·05	2·96	25·86	331					
Calving	88·20	8·30	2·28	27·03	335	111·96	12·25	4·78	34·48	291
1 after	55·64	7·20	1·76	33·15	343					
2 „	56·52	8·75	3·31	30·30	344	111·08	13·00	5·62	36·14	283
3 „	64·44	10·65	5·67	26·08	330					
4 „	45·08	10·15	6·37	20·13	337	97·00	13·30	6·31	28·57	289
5 „	56·52	9·60	5·58	29·27	331					
6 „	55·64	9·65	5·67	30·61	343					
	Cow No. 16.							Calf No. 16.		
1 before	82·92	9·90	2·68	32·43	333					
Calving	83·80	9·80	2·36	29·70	344	130·44	—	5·62	—	—
1 after	71·48	9·75	4·30	29·85	347	119·00	11·80	5·19	45·80	285
2 „	54·76	9·20	5·19	33·15	345					
3 „	59·16	9·40	5·58	29·85	346	116·36	12·70	5·44	40·27	276
4 „	63·56	9·35	4·38	29·13	335					
5 „	70·60	9·30	4·62	25·00	334	125·16	12·80	4·75	46·51	274
	Cow No. 19.							Calf No. 19.		
1 before	63·56	11·55	3·84	31·41	320					
Calving	71·48	10·05	2·61	36·14	315	143·64	12·85	5·23	45·11	276
1 after	57·04	10·00	4·47	34·09	344					
2 „	56·52	9·45	4·41	35·08	326	113·72	12·95	5·15	39·20	259
3 „	53·88	10·65	4·12	—	—					
4 „	53·00	11·05	5·36	36·36	319	105·80	12·55	5·53	36·36	273
5 „	52·28	9·65	4·41	32·97	321					
	Cow No. 20.							Calf No. 20.		
Calving	94·36	10·10	2·79	37·02	327	120·76	12·95	5·00	49·85	301
1 after	68·84	9·75	3·92	35·08	331					
2 „	64·44	10·70	5·44	34·88	338	124·28	13·40	5·36	42·55	291
3 „	67·08	10·75	5·36	34·09	333					
4 „	56·52	10·80	6·43	37·26	331	108·44	14·20	6·03	38·22	298
5 „	53·88	10·95	6·55	35·71	336					
6 „	60·04	10·50	5·04	37·74	339	117·24	13·65	6·25	42·55	293

birth) and are shown on the curves in Fig. 1. On the left-hand side of the curves there is shown the average level of each constituent in the blood of the cows about one month prior to calving, when the cows were dry: these points are joined to the curves by dotted lines. Whilst these values cannot be taken as strictly mathematical averages, the curves clearly indicate the types of change which occur with the different blood constituents.

Discussion of results.

Whilst there are variations in behaviour between individual cows, the results on the whole are fairly uniform in character. A possible exception is Cow No. 12 and it must be noted that this cow was off her food for 2 days after calving. The blood-sugar level of the cows showed a definite and marked increase at the time of parturition, followed by a rapid fall to a low level

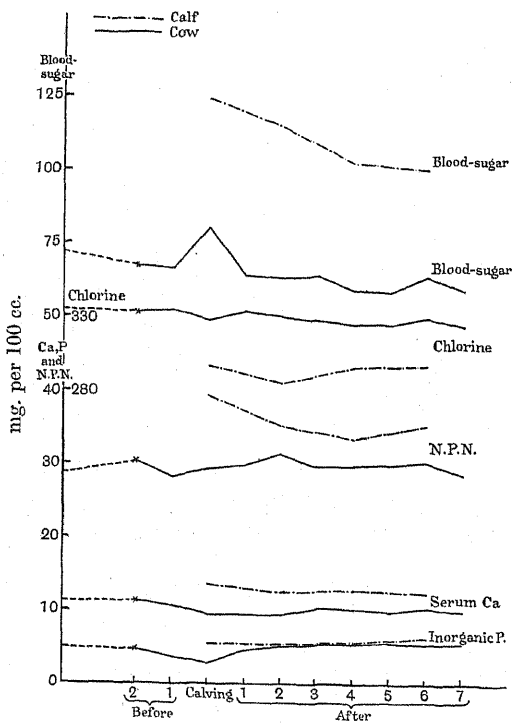


Fig. 1. Changes in the average composition of the blood of the calf and its dam about the time of parturition.

Note. There is a change in the ordinates scale above 40, values 50–125 being the ordinates for blood-sugar.

which was maintained throughout the first week after calving. The rise at calving is of such short duration that it is probably due to emotional hyperglycaemia. On the basis of the views of Kaufmann and Magne [1906] and of Blackwood and Stirling [1932] the lower blood-sugar level after calving may be explained by the requirements of the animal for milk-production.

Serum-calcium underwent in all cases a definite fall either just at or within 24 hours of calving, a fall which, in the majority of cases, was followed by a return to the normal level within the next 4 or 5 days. An exception to this

was Cow No. 5 in which recovery was not apparent within the week. Cow No. 12 was very irregular, which was probably due to the fact, already noted, that she was off her food for 2 days. On the day after calving her serum-Ca, which had dropped at calving, was well up again and this was accompanied by an abnormally high value for the inorganic P (8·42 mg./100 cc.). On the second day the serum-Ca was down well below the calving level after which it slowly rose again, whilst the inorganic P showed a fairly steady decline all this time. It may be noted that this cow was off her food again on the ninth day after calving, and a blood sample drawn then showed a low serum-Ca and a high inorganic P. These results are of interest in reference to the changes in the composition of the blood of the cow, which occur during milk fever. In the light of these observations it would appear that the abnormal fall in the value of serum-Ca during milk fever may be only an exaggeration of the normal physiological change which occurs in the blood of the cow at or immediately after calving.

The most noticeable feature of the data was the unfailing low value for inorganic P just prior to calving, the lowest level usually being reached actually at calving. Cow No. 15 was exceptional in showing a further drop the day after calving, when the inorganic P reached the abnormally low level of 1·76 mg./100 cc., the lowest level we have yet encountered for any healthy cow. This regular fall in blood-inorganic P prior to calving was in most cases a better index of approaching parturition than the outward and visible signs. Normally the inorganic P level rose rapidly after calving and after a peak period at about the fourth or fifth day fell again to about the pre-parturition level.

Whilst individual cows showed considerable fluctuations in the level of chlorine and non-protein-nitrogen, an examination of the data shows no decided tendency in any one direction, the fluctuations being such as could be readily accounted for by variations due to the strain of labour.

A comparison of the values obtained for the blood of the calf with those for the blood of its dam reveals that at birth the calf has a much higher blood-sugar level than that of its dam, the difference being of the order of 30–50 mg. per 100 cc., but although the value falls rapidly and evenly in the first 6 days it still remains well above that of the maternal blood. The calf also starts its life with a high serum-Ca level, higher than the normal level of its dam, and an inorganic P level almost equivalent to the dam's normal. Within the first week the serum-Ca falls slightly while the inorganic P continues to rise steadily and slowly. The chlorine content of the blood of the calf is on an average about 30 mg. per 100 cc. lower than that of its dam, this difference being maintained during the first week. The non-protein-nitrogen level on the other hand is much higher in the calf's blood at birth than in that of the cow.

SUMMARY.

1. Blood-sugar showed a definite rise in the cow at the time of parturition: this was followed by a fall to a low level maintained for at least one week.

2. Serum-Ca fell definitely either just at, or within 24 hours of, calving and returned to normal within 4 or 5 days.

3. A sharp fall in inorganic P just prior to calving was invariably indicative of the onset of labour.

4. The blood of the calf at birth had higher levels of blood-sugar, serum-Ca, inorganic P and non-protein-nitrogen and a lower chlorine level than had that of its dam.

We desire to acknowledge our indebtedness to Mr W. Thomson for assistance with the animals.

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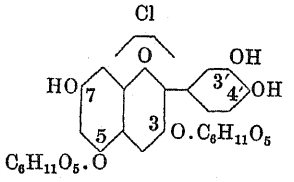
CXCVII. A SURVEY OF ANTHOCYANINS. II.

By GERTRUDE MAUD ROBINSON and ROBERT ROBINSON.

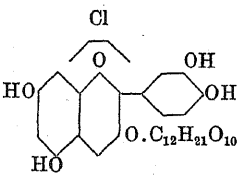
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During the year following the publication of Part I [1931] of this series the progress of synthetical work has been rapid and the main diglucosidic anthocyanins have been synthesised and found to be identical with the natural products. Thus Dr A. R. Todd [1932] in collaboration with one of us, has synthesised hirsutin, malvin, cyanin, peonin and pelargonin chlorides, whilst the preparation of cyanidin 3-cellobioside, cyanidin 3 : 7-diglucoside and 5 : 7-diglucoside has made it clear that mecocyanin and keracyanin are in fact, as already assumed, cyanidin 3-biosides. The established formulae for cyanin chloride (I) and mecocyanin chloride (II) are here reproduced for convenience of reference to the numbering and nomenclature system.



(I)



(II)

The synthesis of cyanidin 3 : 7-diglucoside (MacDowell and Robinson; unpublished results) introduces a matter of which it is necessary to take cognisance, since it is difficult to distinguish this substance from cyanin by reactions in solution unless the p_H can be estimated accurately. Thus the class "3 : 5-dimonoside" may include some 3 : 7-dimonosides and ultimately special investigations will be made in order to determine whether the 3 : 7-isomerides occur in nature or not. In the meantime 3 : 5 is held to include 3 : 7.

Formerly the rhamnoglucosides were not differentiated from the monoglucosides on the one hand or from the diglucosides on the other, but we have now devised a simple test which enables the three classes to be sharply distinguished; we have also simplified the distinction of rhamnose from a simple pentose such as xylose but have not yet applied this to very many cases.

The system adopted in naming the anthocyanins is the following: *saccharide* is the most general term applicable to all sugars, monoses, bioses *etc.*; *glycoside* indicates a single unit of a hexose; *bioside*, a residue of a biose; *dimonoside*, two units of hexose separately combined in different positions of the anthocyanidin

molecule; *pentoseglycoside*, a residue of a biose composed of an unidentified pentose and an unidentified hexose; rhamnoglycoside, a residue of a biose derived from rhamnose and an unidentified hexose.

Naturally we agree that it is very probable that most of the glycosides are glucosides and that most of the pentoseglycosides are rhamnoglucosides but exceptions are known to exist and therefore the more general terminology is retained.

The methods used in the present investigation are the same as those described in Part I but attention must be drawn to a pitfall in connection with the recognition of complex anthocyanins (acylated anthocyanins). We have found that the test for complex character cannot be safely applied unless the anthocyanin is first purified as already described through the picrate or better by a method mentioned below. There is a substance present in crude plant extracts which is capable of condensing with the anthocyanin molecule in hot alkaline solution so that on reconstitution of the pyrylium salt it appears to be diglucosidic. Thus even synthetic monoglucosides, such as oenin or chrysanthemin, can be modified by treatment with hot aqueous sodium hydroxide and an extract of the skins of white grapes (for example) so that their distribution between amyl alcohol and dilute hydrochloric acid becomes that of a diglucoside. The only correction of Part I involved in this relates to the pigment of the copper beech which is really monoglucosidic and which has now been isolated and analysed.

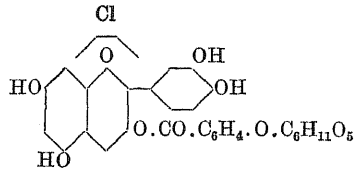
The complex diglycoside test is perfectly satisfactory if applied to the purified pigments and fortunately the high distribution number of these complex pigments makes the purification relatively easy.

Experience of the application of the methods described in Part I has proved their adequacy but exceptional cases will always require special treatment and there is one hitherto unrecognised class of anthocyanins which must be specifically mentioned.

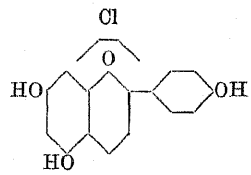
Hydrolysis by 15 % hydrochloric acid under the prescribed conditions is a drastic process and 30 seconds usually suffices to break down the anthocyanin into its constituent parts.

In several cases, however, it appears that sugar residues are eliminated, leaving an acylated anthocyanidin which is further hydrolysed on continued boiling with hydrochloric acid, or, more certainly, by treatment with aqueous sodium hydroxide followed by hydrochloric acid. The normal anthocyanidin reactions are obtained ultimately and the intermediate complex anthocyanidin may be a sparingly soluble precipitate or it may be recognised by special properties. Thus a substance which has been frequently encountered is a 3-acylated cyanidin and this has the curious property that it is largely extracted by the cyanidin reagent (*cyclo*hexanol-toluene 1 : 5) and not at all by the delphinidin reagent (picric acid-anisole-ethylamyl ether). The colour reactions of the anthocyanins which give rise to this 3-acylated cyanidin preclude acceptance of the view that the sugar residues are attached to other

positions of the anthocyanidin nucleus; all the other phenolic hydroxyls, namely those in positions 5, 7, 3′ and 4′ must be free. Consequently the acyl group itself must carry the sugar residues, and this is quite feasible since such acids as *p*-hydroxybenzoic acid, *p*-coumaric acid and protocatechuic acid have been found in association with anthocyanins. Our hypothesis respecting the arrangement of the groups in these cases is illustrated by the expression III.



(III)



(IV)

This sequence of the side-chain groups does not, however, apply to the complex 3 : 5-dimonosides, such as monoardaein, because these give the diglycosides on hydrolysis and we may not substitute more than two of the phenolic hydroxyls on account of the colour reactions. The complex anthocyanidin glucosides, naturally, do not give anthocyanins on alkaline hydrolysis but are at once converted into anthocyanidins.

Some striking cases of the distribution of anthocyanins have been observed. A nasturtium was found to contain a pelargonidin derivative in the flower petals, a cyanidin derivative in the calyx and a delphinidin derivative in the leaves.

Three Pompon dahlias were selected at a nursery and again representatives of the three main types were diagnosed. The pelargonidin analogues of mecocyanin and keracyanin have been repeatedly encountered; they are among the more commonly occurring anthocyanins and we are making arrangements with a view to their isolation in substance.

Several new sources of betanin-like pigments have been found and, of these, *Bougainvillaea* is of special interest because one species appears to contain a monoglycoside of the group. This will greatly facilitate the investigation of what is perhaps the most interesting of all types of anthocyanins.

Some years ago one of us in collaboration with D. G. Pratt, A. Robertson and others synthesised various pyrylium salts allied to the flavones rather than to the flavonols because it appeared probable that these would ultimately be found to be natural products, but until quite recently this speculation was unjustified by the outcome of the present survey. Messrs. Carter's Tested Seeds, Ltd., however, kindly sent us some flowers of *Gesnera fulgens* the colour of which would normally indicate pelargonidin 3-bioside or perhaps a carotenoid pigment. The pigment was soon recognised as an anthocyanin derived from apigeninidin chloride (IV) and owing to the stability and sparing solubility of this salt we were able to obtain about 10 mg. of the pure substance from a mere handful of the flowers. As the material could not be obtained

in quantity we employed both *Gesnera fulgens* and *G. cardinalis* having found that the reactions of the anthocyanins were identical. The apigeninidin chloride was carefully compared with a specimen obtained by the method of Pratt, Robertson and Robinson [1927] and no divergences were observed.

Finally, we draw attention to the great stability of the majority of the anthocyanins in 1 % aqueous hydrochloric acid at the room temperature. Dr E. Fonseka very kindly sent us some specimens from Ceylon in the form of extracts and these gave the same results as freshly prepared solutions, the diglucosides suffering no partial hydrolysis. On the other hand some complex anthocyanins are extraordinarily readily hydrolysed, and the complex anthocyanin in *Centradenia grandiflora* (leaves) is converted into a normal diglucoside by the operation of purification through the picrate. Also some hydrangea anthocyanins and one derived from the chrysanthemum may be cited in this connection. The complex character is lost on merely keeping the solutions.

Experimental.

Notes on methods. The purification of anthocyanins through the picrates is often a very wasteful process and frequently cannot be applied at all; *cyclo*hexanone is the best solvent. An alternative is to take advantage of the large effect of saturation of the solutions with salt on the distribution to amyl alcohol and butyl alcohol. Rosenheim [1920] was the first to suggest the use of butyl alcohol as a solvent for anthocyanins. The anthocyanin can thus be taken up in one of these solvents and returned to dilute hydrochloric acid by addition of a small proportion of benzene or light petroleum and successive extractions with small volumes of 1 % acid. The method can be applied as often as desired and the aqueous solutions can be washed with ethyl acetate or other organic solvent. An example of the process is the isolation of a crystalline anthocyanin from the leaves of the copper beech.

In preparing a solution of an anthocyanidin for the cyanidin reagent test, care must be taken to exclude ethyl alcohol and its derivatives, and to see that the acid concentration is 1 %. This is secured by repeated washing of the amyl alcohol containing the anthocyanidin with 1 % hydrochloric acid before adding benzene and extracting with the acid solution. The amyl alcohol must later be thoroughly removed by washing with benzene.

Test for pentoseglycosides (*rhamnoglucosides and the like*). At very low concentrations of the anthocyanin a distinction between pentoseglycoside and monoglycoside is possible by means of the distribution to amyl alcohol. The colour of the latter tends to become more apparent than that of the aqueous layer in the case of monoglucosides whilst with rhamnoglucosides it is the aqueous layer in which the colour appears to be more persistent. In practice the distinction is very easy to observe.

Similarly a distinction between diglycosides and pentoseglycosides can be obtained at low concentrations by means of the distribution to amyl alcohol.

Both appear almost zero (colourless organic layer) but on the addition of salt to the aqueous layer the pentoseglycoside is largely extracted by the amyl alcohol and the diglycoside distribution is almost unaltered. This applies even to delphinidin derivatives and pelargonidin derivatives.

Tests for rhamnose and aldopentose (xylose). The anthocyanin solutions must be purified as completely as possible by the method mentioned above and by repeated extraction with organic solvents. If anthoxanthins are present it is hardly good policy to conduct this test; the absence of anthoxanthins is indicated by normal, typical alkali-colour reactions of the anthocyanin.

The solution is mixed with an equal volume of concentrated hydrochloric acid and distilled, the distillate being examined for the presence of furfuraldehyde or methylfurfuraldehyde. These can be differentiated by the acetic acid aniline test or by a modification of the naphtharesorcinol test [Tollens and Rorive, 1908; Votoček, 1897]. We have found that the two aldehydes give entirely different reactions when their dilute aqueous solutions are added to a solution of naphtharesorcinol in 5 % ethyl alcoholic hydrogen chloride. The conditions are not critical and may need modification to suit special cases. We have convinced ourselves in this way that the pelargonidin 3-pentoseglycoside in the gloxinia is a rhamnose derivative and we confirmed Nolan and Casey's statement [1931] that their sambucicyanin is derived from an aldopentose and not from a methylpentose.

The following experiment may serve as a guide in applying this very characteristic reaction.

Distil rhamnose or xylose (0·025 g.) with concentrated hydrochloric acid (5 cc.) and water (5 cc.) and collect 5 cc. To 1 cc. of distillate add 5 cc. of acetic acid and 2 cc. of aniline. Xylose gives a very intense crimson and rhamnose gives a much weaker, browner orange-red.

Add 1 cc. of the distillate to 10 cc. of 5 % alcoholic hydrogen chloride containing 5 mg. of naphtharesorcinol. Xylose gives an immediate orange-yellow (without blue tinge), this quickly becomes darker and duller brown and later violet and acquires a most intense green fluorescence. Rhamnose gives at once a stable orange-red, eosin-red in thin layers. The colour does not change in the course of a few hours, but on long standing it changes to reddish-violet and the solution acquires green fluorescence; at this stage the solution from xylose is violet-blue and the fluorescence is much the more intense of the two.

Summary of results.

Acalypha bispida. Hydrolysis for a short time affords an acylated anthocyanidin; hydrolysis for several minutes under the usual conditions, or alkaline hydrolysis, affords cyanidin. The reactions are those of a 3-substituted cyanidin. The distribution number (amyl alcohol) is high and the anthocyanin is probably a 3-acyl cyanidin saccharide.

Acer dissectum atropurpureum, cyanidin 3-monoside; *A. palmatum atropurpureum, A. dissectum versicolor* and *A. campestre Schwedleri* contain the same anthocyanin, all in the leaves.

Achimenes. Flowers of Sutton's "Mauve Queen" give a co-pigmented solution containing malvidin 3 : 5-dimonoside; "Magnet," magenta flowers, contains the same pigment.

Aethionema grandiflora. Bluish-pink flowers contain delphinidin diglycoside with a slight admixture of cyanidin derivative.

Ajuga reptans. Violet-blue flowers contain a complex delphinidin saccharide. Acid hydrolysis gives an acylated anthocyanidin, but if the solution is first treated with sodium hydroxide, hydrolysis by hot hydrochloric acid affords delphinidin.

Allium cepa. The red skins of "Sutton's Blood Red" onion contain cyanidin 3-pentoseglycoside.

Allium narcissiflorum. The violet-red flowers contain a complex (acylated) cyanidin 3-monoside. The original pigment has an abnormally high distribution number and after purification and hydrolysis with sodium hydroxide it yields a cyanidin 3-monoside.

Alstroemeria aurantiaca. Orange-red and orange varieties are chiefly coloured by carotenoids, but these are accompanied by cyanidin 3-bioside.

Amherstii nobilis—pelargonidin 3-pentoseglycoside.

Ampelopsis hederacea—cyanidin 3-monoside in the red leaves.

Anemone pulsatilla. The blue-violet flowers contain delphinidin diglycoside (correction of Part I).

Anthurium scherzerianum. The vermilion leaves and the orange-scarlet flowers are coloured by pelargonidin 3-pentoseglycoside; the leaves also contain a cyanidin derivative in small relative amount.

Antirrhinum majus. The purple leaves of the variety "Defiance" (orange-scarlet flowers) contain pelargonidin 3-pentoseglycoside and the purple leaves of "Monarch" (crimson flowers) contain cyanidin 3-pentoseglycoside. The latter is probably Scott-Moncrieff's [1930] antirrhinin which is identical with the keracyanin of Willstätter and Zollinger [1916]. The flowers of "Monarch" were also examined and the presence of antirrhinin confirmed.

Apium graveolens. Violet-red leaf-stalks of "Sutton's Giant Red" celery appear to contain a complex cyanidin diglycoside, short hydrolysis of which furnishes an acylated anthocyanidin. The peculiar feature is that the distribution number is very low, corresponding to a normal diglycoside. The reactions approximate to those of the 3-bioside type but are not identical with them; for example, the colour with sodium carbonate is violet-blue rather than blue-violet.

Aquilegia alpina. The red-tinged blue flowers contain a delphinidin diglycoside. Another variety, deep violet-blue flowers, gave the same results. There is not much co-pigment in evidence.

Ardisia crispa. The bluish-red berries contain a cyanidin 3-glycoside, the distribution number of which appears to be lower than that of chrysanthemin chloride. As much anthoxanthin is present, a possible explanation is combination of anthocyanin and co-pigment in the aqueous layer.

Aster. A. Amellus var. Riverslea has deep heliotrope or blue-violet flowers containing delphinidin diglycoside. "Colwell Beauty" has a similar but paler shade and the delphinidin derivative in this case is mixed with cyanidin (probably). The Michaelmas daisy "Mrs T. J. Wright," light plum-coloured flowers, is coloured by a cyanidin 3 : 5-dimonoside.

A. sinensis, violet-blue flowers, contains a delphinidin diglycoside contaminated by a methylated derivative or by a cyanidin diglycoside.

Aubrietia deltoides (compare Part I). "Fire King," reddish-violet flowers, contains a 3 : 5-dimonoside of cyanidin.

Begonia. Leaves of several varieties varying from brownish-red to bluish-crimson in colour were all found to contain cyanidin 3-bioside. *B. Margaretacea* and *B. rubrum* gave pure 3-bioside; "Gloire des deceaux" afforded a mixture of bioside and complex diglycoside and some evidence of contamination by the latter was also found in the leaves of the varieties "Professor Grieve," "Gloire des Ardennes" and *B. rex* var. *Versuive,* especially the latter.

Berberis aquifolium. Young leaves, faded leaves and purplish leaves in winter contain a cyanidin 3-pentoseglycoside.

Berberis Darwinii. Purple-blue berries appear to be coloured by petunidin monoglycoside and this is also true of the berries of *B. Stenophylla.* The anthocyanidin contained traces of malvidin or cyanidin or both. On the other hand the orange-red berries of *B. Farreri* and "Winter Cheer" contain a 3-glycoside of cyanidin and the berries of *B. vulgaris* contain the 3-glycoside of pelargonidin. It will thus be seen that the berries of species of *Berberis* embrace an unusually wide range of anthocyanins, all of which are monoglucosidic.

Bertolonia Houtteana. The bluish-red leaves yield an anthocyanin with an abnormally high distribution number. This was purified by the salt-amyl alcohol process and then proved to be a complex malvidin 3 : 5-dimonoside.

Bougainvillaea glabra. The pale pinkish-heliotrope bracts contain a pigment of the betanin group but one yielding acid solutions even bluer in tone than those of betanin. Moreover the distribution number is that of a monoglycoside. The variety "Mrs Butt" contains a diglycosidic pigment of the same group; this closely resembles betanin but its solutions are not quite so blue-toned.

Brassica campestris Rapa. The tops of purple-coloured turnips gave strongly co-pigmented anthocyanin solutions. The pigment proved to be resistant to hydrolysis but tallied with cyanidin 3 : 5-dimonoside in other respects.

Brunfelsia grandiflora, deep heliotrope, contains cyanidin diglycoside with a malvidin derivative.

Caladium bicolor. Crimson leaves, cyanidin 3-pentoseglycoside.

Calceolaria. The bluish-red flowers of the variety "Victoria Prize" contain cyanidin 3-pentoseglycoside.

Camassia esculenta. The violet-tinged blue flowers contain a delphinidin diglycoside.

Campanula muralis, violet-blue flowers, and *C. pusilla,* pure blue flowers, contain a delphinidin diglycoside; the former contains also a second anthocyanin, probably based on cyanidin.

Cattleya labiata Lindl. The magenta flowers give strongly co-pigmented solutions of cyanidin 3 : 5-dimonoside.

Ceanothus veitchianus. Lavender-blue flowers contain delphinidin diglycoside.

Centradenia grandiflora. The bluish-red leaves yield a co-pigmented solution of an anthocyanin having a high distribution number. After purification through the picrate, alkaline hydrolysis afforded malvidin 3 : 5-dimonoside. The pigment is therefore a malvidin complex diglycoside.

Centranthus ruber. The anthocyanin of the rose-pink flowers did not tally with known types in regard to distribution number and alkali-colour reactions; the anthocyanidin is cyanidin.

Cereus grandiflorus. The anthocyanin of the coral-red flowers closely resembles betanin in all its properties; it is perhaps a shade more blue-toned in acid solution.

Cheiranthus Cheiri. The bluish-magenta flowers of the variety "Ruby Gem" give a highly co-pigmented solution of cyanidin 3 : 5-dimonoside. A considerable quantity of this pigment was isolated in the crude condition some years ago from a red wallflower; this has been crystallised and identified as cyanin chloride by careful comparison with an authentic specimen. The Siberian wallflower (*Erysimum asperum*) produces anthocyanin-free flowers.

Chionodoxa Luciliae, blue flowers, contains a complex diglycoside of delphinidin.

Chrysanthemum. Brown-red flowered varieties like "In Memoriam" and "Enton Beauty" appear to contain a complex monoglycoside of cyanidin whilst the blue-red flowered varieties such as "Rose Chochod," "Rona," "Exmouth Pink" and "October Rose" contain a complex pentoseglycoside of cyanidin. In both cases the acyl group is readily eliminated, either by keeping the solutions in 1 % hydrochloric acid or by preserving the petals themselves. The purple leaves of the varieties "Mrs W. H. Webber," "Golden Seal" and "Source d'Or" contain a complex monoglycoside of cyanidin.

Clerodendron fallax. The reddish-orange flowers contain pelargonidin 3-bioside.

Colchicum autumnale. Malvidin 3-bioside.

Columnea Banksii. A hybrid with orange-scarlet flowers was found to contain pelargonidin 3-bioside.

Convolvulus minor, blue flowers, contains a delphinidin diglycoside and traces of methylated delphinidin derivatives.

Cordyline terminalis (Wartelli). The deep bluish-red leaves contain a complex 3 : 5-dimonoside of cyanidin. The distribution number is, however, quite low and the acyl group is only recognised by the fact that in presence of salt the pigment can be extracted from aqueous solutions by amyl alcohol. After hydrolysis by means of sodium hydroxide, good cyanin-type reactions were obtained and the distribution number remained low even when the solution was saturated with salt.

Cornus sanguinea. The blood-red twigs contain a cyanidin 3-saccharide. The tests for complex diglycosidic character gave results difficult to interpret.

Cosmea bipinnata. The magenta flowers gave a highly co-pigmented solution and the anthocyanin is mainly peonidin 3-bioside, but it is mixed with an anthocyanin based on cyanidin and probably also a delphinidin derivative such as malvidin.

Cotoneaster frigida. The red berries contain cyanidin 3-pentoseglycoside.

Cotoneaster Simmonde. Pelargonidin 3-pentoseglycoside and a cyanidin derivative in smaller relative amount.

Crambe maritima. The purple heads of sea-kale gave a highly co-pigmented solution of a cyanidin 3 : 5-dimonoside.

Crataegus. The red berries of *C. coccinea, macracantha, orientale,* contain cyanidin 3-monoglycoside. The flowers of *C. oxyacantha fl. pleno Rosea* also appear to contain a 3-monoglycoside of cyanidin (correction). In dilute solution the apparent diglycosidic character is due to the presence of impurities; after purification the anthocyanin is clearly monoglucosidic. However, the young red shoots were found to contain cyanidin 3-pentoseglycoside.

Crocus vernus. Violet-blue flowers are coloured by delphinidin diglycoside.

Cydonia Japonica. Pink flowers contain a 3-pentoseglycoside of cyanidin with a small relative proportion of a pelargonidin derivative. A variety with salmon-scarlet flowers was found to contain pelargonidin 3-pentoseglycoside.

Cytisus scoparia. The variety "Dorothy Walpole" with brownish blue-red flowers contains cyanidin 3-glycoside.

Dahlia. "Coltness Gem," orange-scarlet, contains pelargonidin 3 : 5-dimonoside and much co-pigment. "Lady Aileen" gave the same results. Pompon "Bacchus" (scarlet), "Glow" (salmon) and "George Ireland" (mauve) gave, respectively diglycosides (3 : 5) of pelargonidin, cyanidin and delphinidin.

Daphne cneorum. Co-pigmented cyanidin pentoseglycoside.

Daucus carota. Purplish tops of "Sutton's Favourite" contain cyanidin 3 : 5-dimonoside.

Delphinium nudicaule splendens. The scarlet flowers are coloured by pelargonidin 3-bioside.

Dianthus barbatus, crimson, was confirmed as cyanidin 3-glycoside (not pentoseglycoside). A salmon-pink was again found to be pelargonidin 3-glycoside, and search for a pelargonidin 3 : 5-dimonoside (found in the carnations) has failed up to the present. On the other hand a very blue-shade magenta (or purple) gave solutions of cyanidin 3 : 5-dimonoside.

Dianthus. "Spark," deep-crimson flowers, contains cyanidin 3-pentoseglycoside. The alpine *Dianthus,* "Bee's Ruby," with bluish-crimson flowers, contains a cyanidin 3-saccharide with distribution intermediate between monoglucosidic and pentoseglucosidic. Among varieties of *D. caryophyllus* we have found that the bluest shaded flowers contain a cyanin-like anthocyanin. Thus "Mrs Hamilton Fellowes" (deep bluish-rose), "Wivelsfield Claret Improved" (intense bluish-crimson and brownish-crimson) and "Homeric" (deep bluish-red) all contain cyanidin 3 : 5-dimonoside.

Dictamnus fraxinella caucasica. The reddish-violet flowers contain a delphinidin pentoseglycoside contaminated by a cyanidin derivative.

Dielytra spectabilis, rose-red, is coloured by cyanidin 3-bioside; a bluer red variety gave the same results but a greater proportion of co-pigment was present.

Dracaena terminalis. Bluish-red leaves of the variety "Lord Wolseley" contained a complex diglycoside based on a mixture of anthocyanidins in which malvidin appeared to predominate.

Erica gracilis. Purplish-red flowers contain a 3-glycoside of cyanidin.

Erinus alpinus. Delphinidin diglycoside.

Erysimum linifolium. Cyanidin 3-bioside with much anthoxanthin.

Fagus sylvatica. The apparent complex diglycoside character of the pigment of the leaves of the copper beech was due to the phenomenon mentioned in the Introduction. The purified anthocyanin is cyanidin 3-monoglycoside.

The substance was isolated in the following manner. An extract of the leaves (collected at the end of May in Oxford) in 1 % hydrochloric acid was saturated with salt and shaken with an equal volume of amyl alcohol. Each 1000 cc. of the amyl alcoholic solution was mixed and agitated with benzene (500 cc.) and 1 % hydrochloric acid (100 cc.) and the separated aqueous solutions were thoroughly extracted with ethyl acetate. The solutions were then saturated with salt and the pigment transferred to butyl alcohol and from this solution to the minimum of 1 %

hydrochloric acid after the addition of sufficient light petroleum (about one-third to one-half vol.). The aqueous solution was mixed with acetic acid (2 vols.) and precipitated with ether (giving a more concentrated solution), the process repeated (syrup) and again repeated (solid precipitate), the crude pigment being then collected by means of a centrifuge. This material was washed with ether and triturated with not too much saturated aqueous picric acid when, on keeping in the ice-chest, the orange-red picrate crystallised almost completely. The substance was collected centrifugally, washed, and converted into chloride by solution in methyl alcoholic hydrogen chloride and precipitation with ether. The salt crystallised at once when 5 % ethyl alcoholic hydrochloric acid was added to its concentrated solution in 0·5 % aqueous hydrochloric acid. It was recrystallised in the same way and obtained in reddish-brown prisms with a weak green glance. (Found in material dried at 110° in a high vacuum over phosphoric anhydride: C, 52·1; H, 4·4. $C_{21}H_{21}O_{11}Cl$ requires C, 52·0; H, 4·3 %.) The colour reactions in a range of buffered solutions of graded p_H [Robertson and Robinson, 1929] agreed for ten solutions exactly with those of chrysanthemin and idaein and in every respect the anthocyanin has the properties of a cyanidin 3-glycoside. Examination of the distribution number, however, indicated that it is substantially idaein rather than chrysanthemin. (Found: 15·5, 15·3 for 5·0 mg. in 25 cc. of *iso*amyl alcohol and 25 cc. of 0·5 % hydrochloric acid, the solvents being previously equilibrated.) At the concentration used, the distribution number of idaein chloride [Willstätter and Mallison, 1915] is about 14 and that of chrysanthemin chloride about 19. The beech pigment, therefore, appears to be idaein mixed with a small proportion of chrysanthemin. A similar mixture of glucoside and galactoside of delphinidin and malvidin was found by Karrer and Widmer [1927, 1] to occur in the bilberry. Finally the pigment was dissolved in 0·5 % hydrochloric acid and 5 % ethyl alcoholic hydrochloric acid added; one-half of this solution was dusted with chrysanthemin chloride and the other half with idaein chloride. The latter crystallised immediately but not the former. The pigment is therefore idaein.

Ficus carica. Cyanidin 3-monoglucoside (fruit).

Fragaria virginiana. The monoglucosidic character of the pelargonidin 3-glycoside of the strawberry was confirmed.

Fritillaria Meleagris. The dull reddish-purple flowers contain a cyanidin 3-bioside, the reactions of the original solution conforming to the standard type. For this reason it seemed worth while to institute a direct comparison with mecocyanin and a solution of this anthocyanin (1·95 mg.) was diluted with 1 % hydrochloric acid until it matched the *Fritillaria* extract (this contained 5·55 mg. in 100 cc.). The solutions were both washed twice with equal volumes of amyl alcohol and the distribution number was then found to be 4·8 in both cases.

Fuchsia triphylla. Cardinal-red flowers contain pelargonidin 3 : 5-dimonoside. The crimson under-surface of the leaves is coloured by malvidin 3 : 5-dimonoside.

Fulminaria augustina azurea. Blue flowers, tinged with pink, contain delphinidin diglycoside.

Gentiana sino-ornata. The rich blue flowers contain a delphinidin complex diglycoside. Karrer and Widmer [1927, 2] found that gentianin, the pigment of *G. acaulis*, is a *p*-hydroxycinnamoyl derivative of a delphinidin monoglucoside.

Geranium ibericum. The violet-blue flowers give very clean extracts (suitable for standards of colour reactions) of malvidin 3 : 5-dimonoside; *G. grandiflorum* gives the same results.

G. armenum, reddish violet, also contains the malvidin 3 : 5-dimonoside but an anthocyanidin with a positive ferric reaction is also present in small relative amount. Since the anthocyanidin recovered after the oxidation test is Fe –, the second pigment is probably not a cyanidin derivative but may be based on petunidin.

G. sanguinea, deep bluish-pink flowers, contains malvidin 3 : 5-dimonoside.

Gesnera fulgens. The reddish-orange flowers contain an anthocyanin which is difficult to extract but is slowly yielded to 1 % hydrochloric acid. The solution is orange-yellow and gives a red-crimson coloration on the addition of sodium carbonate. On hydrolysis the anthocyanidin is obtained in the usual way; it gives yellow acid solutions and its properties at once recalled those of apigeninidin chloride with which it is in fact identical. It was obtained by hydrolysing the anthocyanin in crude extracts of *G. fulgens* and *G. cardinalis* and passing this to butyl alcohol and 0·5 % hydrochloric acid twice. The colour was too orange-red as the result of contamination with a second anthocyanidin and the latter was removed by addition of a few drops of ferric

chloride and shaking the acid solution with air for 2 hours. The anthocyanidin was transferred to amyl alcohol and then to 0·5 % hydrochloric acid (minimum) in the usual manner after addition of light petroleum. The filtered solution was then concentrated in a vacuum and the oxonium salt precipitated as mercurichloride (slender orange-yellow needles). The chloride was regenerated in very dilute solution and finally an aqueous solution containing hydrochloric acid was concentrated and deposited crystals of the anthocyanidin chloride. Solution was again brought about by the addition of alcohol and heating, and the filtered liquid was mixed with 2 drops of concentrated hydrochloric acid and kept in the ice-chest. Orange prisms separated. (Found: C, 58·1; H, 4·3. $C_{15}H_{11}O_4Cl, H_2O$ requires C, 58·3; H, 4·2 %.) The substance was carefully compared with an authentic specimen of apigeninidin chloride and no differences of any kind in the behaviour of the natural and synthetic material could be observed. The colours in alkaline solutions and in alcohol were indistinguishable.

The distributions using 0·5 % hydrochloric acid with mixtures of *cyclo*hexanol and toluene (1 : 2, 1 : 3, 1 : 4, 1 : 5) were identical and characteristic; also noted were distributions to ethyl ether and *iso*propyl ether in the presence of picric acid and to ethyl acetate. The anthocyanin itself tallied closely in distribution number and other properties with apigeninidin 5-monoglucoside which has been synthesised in collaboration with Dr A. R. Todd. A separate account of this work will, it is hoped, be submitted.

The variety "Orange King" also contains an apigeninidin monoglycoside but *G. zebrina discolor* (with yellow carotenoid) contains pelargonidin 3-pentoseglycoside.

G. isoloma hirsutum, orange-crimson and yellow, also contains the pelargonidin 3-pentose-glycoside.

The leaves and spine-like purple hairs of all species of *Gesnera* examined contain a diglycosidic anthocyanin based on an unidentified anthocyanidin. It is Fe+ and probably not 3-substituted but it is not luteolinidin as was at first suspected. It may be the delphinidin of the flavone series and the synthesis of this substance will be undertaken.

Gladiolus. The purplish-red outer parts of the corms contain pigments which are not readily extracted by 1 % hydrochloric acid even on heating. When obtained in solution the pigment was found to be cyanidin 3-bioside but it may not exist in this form in the bulbs. Corms of "Jacob von Beeren" (purple flowers) and "War" (scarlet flowers) gave the same results, but the latter contained much anthoxanthin and the former very little.

Gloxinia. Carter's "Invincible Pride" and a bright cardinal-red flowered variety contain pelargonidin 3-rhamnoglycoside. The tests for rhamnose were carried out on the anthocyanin purified by the butyl alcohol process (thrice transferred).

Gynura aurantiaca. The bright purple hairs on the leaves contain a malvidin 3 : 5-dimonoside but the anthocyanin required drastic purification before the usual alkali-colour reactions could be observed.

Hedera helix. The purple stems and deep purplish-black berries contain a cyanidin 3-bioside; the same is true of the purple stems of a variety "Silver Irish."

Helianthemum vulgare. A pink-flowered variety, also "Rosy Gem" (bluish-red) and "Ben Venue" (pinkish-orange) were found to contain cyanidin 3-glycoside but the last named is coloured in part by a second anthocyanin tending to a bluer shade in the sodium acetate-amyl alcohol test of the anthocyanidin.

Helichrysum bracteatum. The brown-red to bluish-crimson flowers do not readily yield their anthocyanin and the petals were heated with 1 % hydrochloric acid. The pigment is a cyanidin monoglycoside but the usual colour reactions were too much obscured to allow of satisfactory allocation of sugar-position.

Heuchera sanguinea. The light red, slightly blue-tinged flowers contain a cyanidin complex 3-bioside. It yields an acylated anthocyanidin when hydrolysed for a short period.

Hibiscus rosa Silensis var. *Cooperii.* The bluish-red leaves contain cyanidin complex 3-saccharide and furnish an acylated anthocyanidin on careful hydrolysis.

Hieracium lima. The spotted leaves contain cyanidin 3-glycoside.

Hyacinthus. The purplish bulbs of the varieties "King of the Blues," "Lady Derby" and "Roi des Belges" contain an anthocyanin which is very difficult to extract by means of 1 % hydrochloric acid. Its distribution number approximates to that of an anthocyanidin. The

anthocyanidin is cyanidin and after hydrolysis with sodium hydroxide the anthocyanin is cyanidin 3-pentoseglycoside. The alkali-colour reactions of the original are, however, too blue for exclusive 3-substitution and it should therefore be described as a complex cyanidin 3-pentoseglycoside and it probably bears an acyl group in position 5 or 7 of the phloroglucinol nucleus.

The pink flowers of "Lady Derby" contain a complex pelargonidin 3 : 5-dimonoside (salvianin-monardaein type of anthocyanin). "Pink Queen" and "Ella" (pink) gave the same results as "Lady Derby." If the petals are kept in the air for a week or if the solution in 1 % hydrochloric acid is kept for the same period, the acyl group is removed, the anthocyanin becomes much more readily soluble in 1 % hydrochloric acid and is no longer complex. The distribution and reactions then correspond to a normal pelargonidin 3 : 5-dimonoside. The flowers of "King of the Blues" (hot-house grown) contain a complex diglycoside of delphinidin which is relatively stable and the distribution number is unchanged when the solution in 1 % hydrochloric acid is preserved. On the other hand, the same variety grown in the open (at a later season) seems to produce a normal diglycoside with a very low distribution number.

Hydrangea hortensis. The varieties "Deutschland" and "Parzival" (pink) were found to contain a complex pentoseglycoside of delphinidin when examined in May. The flowers had probably been developed under hot-house conditions; later in the open garden the colour remained the same but the complex character had disappeared. Blue-flowered varieties "Mme A. Riverain" and "Vice-President Truffant" were coloured by delphinidin pentoseglycosides from the commencement. An intermediate shade *H. Rubis* had an intermediate distribution early in the season.

The purple leaves of "Vice-President Truffant" and the adjoining stems contain a cyanidin 3-bioside.

The purple colour developed in the leaves (var. Marechal Foch) by frost or starvation is a mixture of cyanidin 3-glycoside and cyanidin 3-bioside. These were separated and examined independently.

The purplish black stem of *H. cynoclada* gives a highly co-pigmented solution of cyanidin 3-bioside. This is possibly complex since unusually prolonged hydrolysis was necessary in order to obtain the anthocyanidin.

Crystalline acids occur free in hydrangea flowers. Early in the season we obtained one, M.P. 200°, from *H. rubis*; colourless flat needles (Fe –). Attempts to obtain more of this at a later period gave an acid (Fe –) crystallising from 1 % hydrochloric acid in colourless, hair-like needles, M.P. 179°. (Found: C, 60·6; H, 5·5 %.) This substance has not yet been identified.

Ilex Shepherdii (*I. aquifolium* var. *Hodginsii*). The red berries were extracted without bruising and contain a pelargonidin 3-pentoseglycoside and very much anthoxanthin.

Impatiens Holstii. The orange-scarlet flowers contain a pelargonidin complex 3 : 5-dimonoside, closely resembling monardaein. Another variety with deep bluish-rose flowers contains peonidin complex 3 : 5-dimonoside.

Impatiens biflora. The young red leaves contain cyanidin 3-pentoseglycoside.

Ixia orateroides. A reddish-violet variety is coloured by petunidin diglycoside, probably mixed with derivatives of malvidin and delphinidin.

Jacaranda ovalifolia. Delphinidin diglycoside.

Kochia tricophylla Childsii. This plant was examined when the autumnal colouring of the leaves had been produced by starvation. The foliage then contained a colouring matter of the betanin-class differing but little in its reactions from the beet pigment.

Lathyrus odoratus. Mr L. H. Stone of the John Innes Horticultural Institution has kindly submitted to us a number of sweet peas of interest from the point of view of genetics. We comment on the chemical aspect only.

"Colorado," orange, pelargonidin 3 : 5-dimonoside almost free from co-pigment; "Beatall," bluish-red, highly co-pigmented pelargonidin 3 : 5-dimonoside; "Charm," deep bluish-red, co-pigmented 3 : 5-dimonoside of pelargonidin; as in "Colorado" the distribution suggests admixture with a pentoseglucoside or other anthocyanin with a higher distribution number; "Ascot," bluish-pink, very strongly co-pigmented pelargonidin 3 : 5-dimonoside; "Flamingo," orange-scarlet, mixture of pelargonidin 3 : 5-dimonoside and pelargonidin 3-bioside; "Wonderful," slightly bluer scarlet than the last, the same mixture of anthocyanins; "Damask Rose," bright

carmine, peonidin 3 : 5-dimonoside with co-pigment; "Honour," crimson, peonidin 3 : 5-dimonoside with less co-pigment and making the closest approach to a pure anthocyanin in the peonidin group; "Miss Hunt"—wings, magenta-blue, highly co-pigmented peonidin 3 : 5-dimonoside mixed with a smaller proportion of a diglycoside derived from a methylated delphinidin derivative and with one derived from cyanidin or petunidin; a mixture of malvidin and cyanidin derivatives or petunidin alone would fit the facts; "Miss Hunt"—standard, bluish-wine-red, contains the same anthocyanin as the wings but it is far less co-pigmented; "Pinkie," blue-pink, highly co-pigmented peonidin and malvidin 3 : 5-dimonosides; the flavone glycoside has powerful basic properties and gives an intense yellow colour with moderately concentrated hydrochloric acid; "Lord Nelson," deep violet-blue, petunidin diglycoside with a small proportion of malvidin derivative—possibly delphinidin in small amount but no cyanidin; "Purple," "Purple with modified wings" and "Purple Invincible" give similar results and contain, chiefly, petunidin diglycoside with rather more malvidin derivative than was found in "Lord Nelson"; "Copper,' coppery red-violet, also petunidin diglycoside with a little malvidin (less than the purples) and possibly some delphinidin; "Dobbie's Maroon" gives almost the same results as the purples but it is not strongly co-pigmented. *L. tingitanus*, deep violet, co-pigmented malvidin 3 : 5-dimonoside.

In connection with the secondary anthocyanins of the sweet peas it will be necessary to institute experiments on a somewhat larger scale in the more interesting cases; we are unable to detect a slight admixture of delphinidin in petunidin.

Ligustrum vulgare. The dark purple berries contain an anthocyanin which on hydrolysis in the usual manner gives a complex anthocyanidin, very sparingly soluble in dilute hydrochloric acid. This is hydrolysed by aqueous sodium hydroxide to an anthocyanidin tallying with delphinidin except that the colour in the amyl alcohol-sodium acetate test was much too red; also the anthocyanidin was not completely extracted by ether-picric acid. It is possible that the hydrolysis was accompanied by side reactions.

Reinvestigation (after some months, hence the possibility of changes in the solution) showed that the anthocyanin was separable by the amyl alcohol-salt process into monoglucosidic and diglucosidic fractions. The former was found to be based on petunidin, the latter on delphinidin. It was certainly unfortunate that Grafe [1906; 1909; 1911] was led to select the berries of *Ligustrum* as material for his attack of the problem of isolation of anthocyanins but he appears to have obtained crystalline pigments.

Lilium lancifolium rubrum. The white flowers with bluish-red streaks contain petunidin pentoseglycoside and the anthers contain cyanidin pentoseglycoside probably in a solid form as the solution in 1 % hydrochloric acid is produced at once. It is particularly clean and if these anthers were readily accessible in quantity they would form a ready source of (probably) keracyanin.

Crimson bulbs of *L. l. Melpomene* contain a mixture of cyanidin 3-bioside and cyanidin 3-pentoseglycoside. *L. tigrinum*, orange flowers, was found to contain much carotenoid and petunidin pentoseglycoside.

Lilium umbellatum erectum and *L. umb. splendidum*, orange-red and scarlet flowers respectively, contain cyanidin 3-pentoseglycoside.

Limonium sinuata rosea. The pink flowers contain a delphinidin pentoseglycoside. *L. Suworowie*, rose-pink, is coloured by malvidin 3-glycoside together with a small proportion of a petunidin or delphinidin derivative.

Lithospermum prostratum. The bright blue flowers appear to contain an almost homogeneous petunidin diglycoside.

Lobelia erinus. The variety "Crystal Palace," blue, is coloured by a delphinidin diglycoside.

Lonicera sempervirens. Cyanidin 3 : 5-dimonoside. The bright amethyst berries of *L. nitida* contain a cyanidin 3-bioside.

Lotus corniculatus (bird's foot trefoil). The young buds are yellow, tipped bright red and contain cyanidin 3-glycoside.

Lycesteria formosa. The brownish-red berries and calyx contain cyanidin 3 : 5-dimonoside.

Lychnis dioica (red campion). The flowers contain a mixture of cyanidin 3 : 5-dimonoside and delphinidin diglycosides and the stems and calyx contain a monoglycoside of delphinidin with preponderating cyanidin 3-glycoside.

Lychnis viscaria. The bluish-pink flowers contain a mixture of diglycosides of cyanidin and delphinidin; a "Tom Thumb" variety gave similar results.

Magnolia Sennei also *Alexandrina* and *Rustica rubra*, reddish-violet to bluish-pink flowers, appear to contain peonidin 3 : 5-dimonoside. The anthocyanin is diglycosidic and the anthocyanidin is peonidin but the colour reactions are rather obscured, even after purification, by the presence of anthoxanthins.

Meconopsis Baileyi (blue Himalayan poppy) is coloured by cyanidin 3 : 5-dimonoside.

Meconopsis cambrica fl. plena. The orange flowers contain pelargonidin 3-bioside.

Metrosideros speciosus (Australian bottle-brush tree). The crimson flowers contain cyanidin 3 : 5-dimonoside with an admixed delphinidin diglycoside.

Musa coccinea. Pelargonidin 3-glycoside.

Muscari (grape hyacinth). Delphinidin diglycoside.

Naegelia. "Walmsgate Beauty" has a dull blue-crimson leaf coloured by a cyanidin 3-bioside.

Nemesia strumosa. "Suttonii," pink and crimson hybrids, contain a cyanidin 3-bioside. "Blue Gem" contains delphinidin pentoseglycoside. The orange shades are largely due to carotenoid pigments.

Nicotiana. "Sutton's Scarlet" contains a delphinidin pentoseglycoside.

Nymphaea gloriosa, bluish-crimson, is coloured by a delphinidin glycoside, possibly complex. *N. stellata odorata*, blue, contains a complex diglycoside of delphinidin. *N. Zanzibariensis*, pinkish-mauve, contains a delphinidin glycoside.

Ononis spinosa. The pink flowers contain a delphinidin pentoseglycoside.

Orchis mascula, violet, is coloured by cyanidin 3 : 5-dimonoside.

Paeonia. The young red leaves contain a cyanidin 3-bioside but the difficulty of hydrolysis and the formation of an acylated cyanidin indicate the presence of some complex diglycoside.

Papaver nudicaule. It has been confirmed that the anthocyanin of the orange flowers is mainly pelargonidin 3-bioside and not pentoseglycoside. The oriental poppies also contain biosides, but pentoseglycosides occur in some of the Shirley varieties.

Pelargonium. We have directed our attention to the nature of the anthocyanin in the bluer red varieties and find that it is usually a malvidin derivative. The salmon-pink Zonal Pelargonium, "Kovaleski," contains practically pure pelargonidin 3 : 5-dimonoside (pelargonin). Flower petals of "Henry Jacoby" contain chiefly pelargonin with some malvidin 3 : 5-dimonoside, but no cyanidin derivatives. The "Versailles" (Regal) variety "Madame de Monier," is likewise coloured by a pelargonin-malvidin 3 : 5-dimonoside mixture and cyanidin is absent. Petals of the flowers of *P. Veitchianum*, reddish-violet, *P. Bertiana*, reddish-mauve and *P. Cucullatum*, reddish-mauve, contain chiefly malvidin 3 : 5-dimonoside.

On the other hand, the flower stalks and calyx of "Henry Jacoby" contain cyanidin 3 : 5-dimonoside and the leaves of "Paul Crampel" also contain a cyanidin diglycoside.

Pentstemon heterophyllus, pure blue occasionally tinged pink, gives a highly co-pigmented extract containing a delphinidin diglycoside. *P. platyphyllus*, lilac, and *P. Garrette*, bright blue, gave similar results.

A violet-flowered variety contained a mixture of monoglycosidic and diglycosidic delphinidin derivatives and these were separated and examined independently.

Petunia. "Rosy Morn," deep pink, is coloured by peonodin 3 : 5-dimonoside.

Phaseolus multiflorus vulgaris. The flowers of the Scarlet Runner bean are coloured by pelargonidin 3-bioside. The reddish-purple seeds were placed in hot water for a short time and the coloured exterior parts then removed and extracted by means of 1 % hydrochloric acid. The anthocyanin is apparently a complex cyanidin pentoseglycoside but as it gives a nearly pure blue colour with sodium carbonate, there is some peculiar feature in connection with the arrangement of the attached groups. The brownish-violet seeds of the "Canadian Wonder" bean contain pelargonidin 3-glycoside.

Phlox Drummondi. The examination of some hybrids was rendered difficult by the presence of co-pigments. Crimson flowers contain a petunidin glycoside. Bluish-crimson flowers also contain petunidin glycoside mixed with monoglucosidic derivatives of malvidin and delphinidin. Violet flowers, also magenta flowers, contain delphinidin diglycoside along with a malvidin (and possibly petunidin) derivative.

Plumbago carpentae. The bright blue flowers contain malvidin and delphinidin (or petunidin diglycosides and the reddish leaves contain cyanidin 3-glycoside or 3-pentoseglycoside.

Poinsettia euphorbia pulcherrima. The red leaves contain cyanidin 3-pentoseglycoside.

Primula. Malvidin 3 : 5-dimonoside has been found in *P. Burmanica,* bright violet-red, *P. vulgaris,* blue and lavender-blue, also in the variety *Wilsonii,* pale pink-heliotrope, in *P. Veitchii,* deep bluish-red, in *P. Juliana,* deep reddish-plum coloured, mixed with hirsutidin 3 : 5-dimonoside. The latter anthocyanin (very probably hirsutin) occurs in *P. denticulata,* mauve, and also in *P. frondosa* and *P. rosea.* "Mrs Neale" gives ambiguous reactions but is probably coloured by a malvin-hirsutin mixture.

P. Japonica (Miller's crimson) seems to contain a malvidin pentoseglycoside. *P.* "Aileen Aroon," brownish-red, is coloured by a mixture of malvidin diglycoside and pentoseglycoside.

Very interesting cases are those of *P. Forrestii,* violet, and *P. Waltoni,* deep brownish-violet, for these are coloured by malvidin 3 : 5-dimonosides in which, from the distribution phenomena, one of the sugars, or even both of them, should be a pentose or methylpentose. This would constitute a novel type since the pentoseglycosides have only been observed in the 3-substituted anthocyanidins.

Among the primulae coloured by hirsutidin 3 : 5-dimonoside is *crispata* which has blue-violet flowers, more blue-toned than hirsutin itself can possibly be at any p_H. This is an example of the influence of a co-pigment of the flavone glucoside type and the phenomenon can be reproduced *in vitro* with synthetic materials.

P. Cockburniana, orange, contains cyanidin 3 : 5-dimonoside; some brownish-red flowered Lisadell Hybrids were also found to contain mixed cyanin- and malvin-like anthocyanins. An orange-red *P. Bulleyana* contained cyanidin 3-bioside and it thus appears that the primulae contain a wide range of anthocyanins. They may also be almost free from these pigments as in the case of *P. Bulleyana,* yellow flowers, which contains only a trace of a malvin-type anthocyanin situated at the base of the corolla.

Prunus communis. Skins of the large blue Californian plum contain cyanidin 3-pentoseglycoside; those of the red South African plum and the light red Gaviota plum contain cyanidin 3-glycoside. This contrast between red and blue-coloured plums appears to hold generally.

P. persica appears to contain cyanidin 3-glycoside in the skins.

P. insititia (damson) has cyanidin 3-pentoseglucoside in the skins of the fruit. The Japanese cherry "Shidaro Sakura" has pink flowers coloured by a cyanidin 3-bioside.

Punica granatum. The pigment in the pomegranate (Californian origin) seems to be delphinidin diglycoside. All the tests indicated delphinidin but the colour of the anthocyanidin with amyl alcohol and sodium acetate was too red-toned. Nevertheless it must be delphinidin or a new anthocyanidin and most probably the former.

Pyracantha coccinea Lalandei. Cyanidin 3-pentoseglycoside.

Pyrus acerba floribunda. This bright red crab-apple contains cyanidin 3-glycoside. *P. acerba Aldenhamensis,* pink flowers, also contains cyanidin 3-glycoside.

Ramondia pyrenaica. The violet-blue flowers contain much anthoxanthin and a delphinidin diglycoside.

Rhododendron. Hardy species derived from *R. ponticum*: cyanidin 3-glycoside is found in most rose-red varieties.

R. yannense, bluish-red flowers, co-pigmented cyanidin 3-glycoside. "G. A. Simms," Doncaster, Ascot Brilliant and *R. Thomsoni,* crimson flowered, also contain cyanidin 3-monoside. "Charles Dickens" with flowers of a very blue-toned red shade, contains cyanidin 3 : 5-dimonoside and intermediate shades between *R. Thomsoni* and this are found to contain mixtures of the cyanidin mono- and diglycosides. Such are "William Austin," *R. arboreum coccineum* and "Mrs Thistleton Dyer." The anthocyanins are readily separable by the salt-amyl alcohol method and the bluer the shade of the red flowers the higher the proportion of cyanin-type anthocyanin was found to be. Thus the rhododendrons afford a further example of the correlation between the colour of flowers and the nature of the sugar side-chains. In any particular series of anthocyanins (all derived from the same anthocyanidin) it is generally true that the monoglycosides, pentoseglycosides and diglycosides make a series of increasing blueness of tone of red flowers.

In the case of the rhododendrons, however, the presence or absence of co-pigments is an

additional factor having a large effect and in several cases it was found that a bluer red rhododendron contained less diglycoside than one not so blue-red because the bluer red flower also contained a higher relative amount of anthoxanthin, probably flavone glycoside in character.

In the Azalea section, *R. indicum*, "Mme van der Cruyssen," bluish-red flowers, contains cyanidin 3 : 5-dimonoside. The variety "Hindu Girl" and a similar unnamed variety were found to contain a mixture of cyanidin 3-glycoside and cyanidin 3 : 5-diglycoside. In each case the pigments were separated and examined independently. The occurrence of pure cyanidin 3-glycoside in salmon-red azaleas has been confirmed (not pentoseglycoside).

Violet and purple rhododendrons contain methylated delphinidin derivatives. *R. splendens*, purple, is coloured by petunidin diglycoside (trace of pentoseglycoside, possibly) containing a small relative amount of malvidin diglycoside.

R. Augustini (*orestrephis* is similar), blue-violet, contains a mixture of cyanidin or petunidin and malvidin 3 : 5-dimonosides, the latter predominating. *R. Californicum*, reddish-violet, is coloured by malvidin 3 : 5-dimonoside with a trace of a cyanidin or petunidin derivative. "Purple Splendour" was found to contain chiefly petunidin diglycoside and "Royal Purple" contains diglycosidic anthocyanins derived from malvidin (chiefly) and petunidin or delphinidin.

Rhodora canadensis (syn. *Rhododendron Rhodora*) contains a delphinidin glycoside in the heliotrope flowers and cyanidin 3-pentoseglycoside in the leaves (autumn).

Rhoco discolor. The purple leaves contain cyanidin 3-bioside.

Ribes sanguineum. The rose-pink flowers of the flowering currant contain two anthocyanins which are readily separable by means of amyl alcohol (without salt). The fraction more soluble in amyl alcohol has almost the distribution number of an anthocyanidin but it is not extracted by ethereal picric acid from aqueous solutions. The anthocyanidin is cyanidin and the anthocyanin is evidently of a novel type. It gives a pure blue solution in aqueous sodium carbonate and on hydrolysis by hydrochloric acid evidence of step-wise removal of more than one group was obtained. Hydrolysis by means of alkalis appeared to give anthocyanidin and some normal monoglycoside. Further investigation on a larger scale is necessary. The more soluble anthocyanin is cyanidin 3-pentoseglycoside.

R. grossularia. The flowers of the gooseberry contain cyanidin 3-pentoseglycoside.

Rosa. The young red leaves of roses contain cyanidin 3 : 5-dimonoside and the haws contain no anthocyanin.

Rosmarinus officinalis. The flowers contain a complex diglycoside of delphinidin.

Rubus fruticosus. The stems and leaves of the blackberry, gathered in winter, contain cyanidin 3-saccharide.

Saponaria vaccaria. The pink flowers contain co-pigment and a cyanidin 3-bioside.

Saxifraga decipiens bathoniensis. The red flowers contain two anthocyanins of which the more soluble in amyl alcohol appears to be a cyanidin complex saccharide. The complex anthocyanin can even be removed by extraction of an aqueous acid solution with *cyclo*hexanone. The anthocyanin, more soluble in dilute hydrochloric acid, is a cyanidin 3-bioside (not tested for pentoseglycoside) which is unusually unstable in alkaline solution. The saxifrage "Sir Douglas Haig" contains a complex anthocyanin which gives a complex anthocyanidin on partial hydrolysis. It is a 3-acylated-cyanidin saccharide (the nature of the sugar groups cannot be estimated in such cases) and this clear case may perhaps give a clue to that of the flowers of the currant (Ord. Saxifragaceae).

Saxifraga cordifolia purpurea (*Megasea cordifolia*). Strongly co-pigmented cyanidin 3 : 5-dimonoside.

Scilla nonscripta. The blue-bell pigment is a delphinidin diglycoside, and a pink-flowered variety contained cyanidin 3-bioside. The flowers of *S. nutans* also contained delphinidin diglycoside and the bulbs are coloured by a complex diglycoside of cyanidin.

Scutellaria violaceae. The violet-red flowers give a highly co-pigmented extract and the anthocyanin is a complex diglycoside of delphinidin.

Sedum spectabilis. Malvidin 3-pentoseglycoside.

Sempervivum arachnoideum. The pink flowers contain malvidin pentoseglycoside mixed with a derivative of another anthocyanidin (Fe+).

Senecio. The cineraria "Rainbow," bright crimson, contains a cyanidin diglycoside which has unusual colour reactions. The violet coloration with sodium carbonate does not give a clear blue on the addition of sodium hydroxide. This suggests substitution in position 3′ and it is therefore of interest that a ferric reaction could not be obtained with the anthocyanin itself. The anthocyanin may be cyanidin 3 : 3′-dimonoside but this is only a provisional hypothesis and further investigation is necessary. The anthocyanin is definitely diglucosidic and it is unlikely that it can be a complex diglycoside.

Solanum Melongena. The purplish skin of the fruit (aubergine) contains delphinidin pentoseglycoside.

Solanum tuberosum. "Sutton's purple Edgecote potato" contains malvidin 3 : 5-dimonoside with a little of a cyanidin derivative. The Congo potato (coloured through the tuber) contains a complex malvidin 3 : 5-dimonoside, the distribution number of which is similar to that of a monoglucoside. On keeping the solution in 1 % hydrochloric acid for 3 months, the acyl group was eliminated and the anthocyanin then gave the reactions and distribution properties of malvidin 3 : 5-dimonoside. The variety "Mr Bresce," pink, and "Cardinal," bluish-pink contained pelargonidin 3-pentoseglycoside and 3 : 5-dimonoside, respectively.

S. pseudocapsicum has berries devoid of anthocyanin and this is also true of *S. dulcamara* but in this case (Bitter Sweet) the stems adjoining the berries contain a delphinidin derivative.

Sophronitis grandiflora. The flowers of this orchid are coloured by pelargonidin 3-bioside.

Spathodea nilotica and *Sterculia lanceolata.* Pelargonidin 3-saccharide with a distribution intermediate between monoglucosidic and rhamnoglucosidic.

Streptocarpus Wendlandii, violet, contains malvidin 3 : 5-dimonoside. The hybrids "Brightness," red, and "Vivid," deep scarlet, contain pelargonidin 3-rhamnoglycoside. Sutton's "Giant" bluish-pink, contains peonidin 3 : 5-dimonoside and a magenta-flowered variety and also Sutton's "Giant," blue, contain malvidin 3 : 5-dimonoside.

Syringa. "Madame Lemoire," light heliotrope, is coloured by delphinidin pentoseglycoside in association with co-pigment. "Congo," reddish-purple, contains the same anthocyanin but relatively to the latter a smaller proportion of co-pigment. Other unnamed species have been examined and the anthocyanin was always found to be the same; the colour variation is due to changes in the concentration of the anthocyanin and of the co-pigments.

Thunbergia. The orange flowers of a hybrid were found to be devoid of anthocyanin but the dark purple corolla tube contained cyanidin 3-glycoside.

Thymus coccineus, bright reddish-violet flowers, contains cyanidin 3 : 5-dimonoside.

Tradescantia Zebrina pendula. The bright purple leaves contain cyanidin 3-bioside along with a delphinidin derivative. *T. virginica*, bluish-violet hybrid, is coloured by petunidin diglycoside.

Tropaeolum Majus. The diglucosidic character of the anthocyanin in the common orange climbing nasturtium has been confirmed and it is from this source that we hope to isolate pelargonidin 3-bioside in substance. *T. M. nana*, "Empress of India," orange-scarlet, has pelargonidin 3-bioside in the petals, cyanidin 3-bioside in the sepals and delphinidin diglycoside in the leaves. The variety "King Theodore," deep brownish-red flowers, contains delphinidin diglycoside in both petals and sepals. The occurrence of delphinidin in these flowers and leaves was not anticipated.

Verbascum Phoeniceum. The dark, dull purple flowers contain peonidin 3-glycoside. This anthocyanin is therefore of the type of oxycoccicyanin [Grove and Robinson, 1931] and may be identical with it.

Veronica Teucrium rupestris, deep blue flowers, contains a delphinidin diglycoside; *V. chamaedrys* contains a delphinidin glycoside.

Viburnum Tinus Laurustinus. The small hard blue berries were extracted by means of hot 0·5 % hydrochloric acid. The anthocyanin is cyanidin 3-glycoside.

Viola odorata. The single violet "Princess of Wales" contains a complex saccharide of delphinidin. A viola known to be a cross between *V. Bosniaca* and *V. cornuta* and possessing flowers having the bluish-pink colour of the former, was found to contain cyanidin 3 : 5-dimonoside. The brownish-red flowered viola "Arkwright Ruby" contains cyanidin 3-pentoseglycoside in the greater part of the petals and in the dark central portions this is mixed with some cyanidin 3 : 5-dimonoside. The colour is highly co-pigmented. Violanin [Willstätter and Weil, 1916] which

is delphinidin rhamnoglucoside has been found to be the characteristic constituent of dark purple violas; the pure blue flowered kinds contain delphinidin diglycoside.

Vitis vinifera. The skins of a red South African grape, "Hanefoot," contain cyanidin 3-glycoside. "Colmar" grapes have a colour intermediate between this and the bluest grapes but the anthocyanin is oenin.

The leaves of *V. discolor* contain a malvidin diglycoside.

Wistaria chinensis. The flowers are coloured by diglycosides of methyldelphinidins, probably largely petunidin.

Zinnia. Bluish flame-coloured flowers contained pelargonidin 3 : 5-dimonoside and a variety with orange flowers gave evidence pointing to the same pigment, mixed with a small proportion of a peonidin or malvidin derivative.

Co-pigments.

Throughout our work we have again observed that the acid extracts of flowers are almost always more blue-toned than are the solutions of the pure anthocyanins and the phenomenon of co-pigmentation is most in evidence when the presence of anthoxanthins can be detected in other ways. The common co-pigments are the tannins and the flavone and flavonol glycosides and we have been able to reproduce most of the effects with these. In general, pelargonidin glycosides, especially 3-biosides, are not greatly co-pigmented, but there are some noteworthy exceptions to this statement (phlox, for example). A solution of tannic acid is a useful auxiliary for the detection of malvidin derivatives because the colour change is much more striking than with peonidin derivatives.

It was stated in Part I that rutin has a feeble co-pigment effect, but it was suggested that this might be due to insolubility. Actually rutin and quercitrin are powerful co-pigments.

It is of interest that papaverine and quinaldine exert a strong blueing and intensifying influence on the colour of oenin, chrysanthemin and malvin, whereas laudanosine and quinoline have very little action.

There is evidence that co-pigmented anthocyanins *in vivo* as well as *in vitro* are stabilised, for example, against the decomposing action of light and experiments on this phenomenon are in progress. It is perhaps on this account that the anthocyanins collected in winter, having, as it were, proved their power to survive, are often very difficult to investigate by reason of the large admixture with other substances. We have also reason to believe that complex anthocyanins tend to be formed when the pigment remains *in situ* for long periods.

We are greatly indebted to the authorities of the Royal Botanical Gardens, Kew, the Botanical Gardens, Oxford, the John Innes Horticultural Institution, Merton, for advice and material and our thanks are also due to Messrs Sutton of Reading, the Brookside Nurseries, Oxford, Messrs James Carter and Co., G. H. Dalrymple, Esq. of Bartley, Messrs Waterer, Sons and Crisp, Messrs B. Ladhams, Limited, Messrs J. and A. McBean, and Messrs Allwood Bros. for generous assistance. Dr E. Fonseka kindly collected some specimens in Ceylon and we are greatly indebted to him.

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CXCVIII. THE DETERMINATION OF SMALL QUANTITIES OF IODINE IN BLOOD.

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(*Received August 23rd, 1932.*)

Since the publication of von Fellenberg's method [1923] for the determination of small quantities of iodine, a very large number of new methods and modifications have been described, some of which claim a surprising degree of accuracy. The multiplicity of methods is sufficient to show that none of them is really satisfactory.

The von Fellenberg method includes two methods for the final determination, colorimetric and titrimetric. We have attempted to produce a method which is free from some of the objections which can be raised against the older ones. While it can be said that the colorimetric method here described, which was devised by one of us (W. H. H.), is satisfactory within the limits of such a method, the titrimetric method has not been quite completed; owing, however, to the impossibility of further collaboration it seemed desirable to record such observations as we have made.

Colorimetric method.

Ashing. Most of the previous workers have not laid down any very strict regulations as to the control of temperature necessary during ashing of the blood, but it became apparent early in the work that this would require careful attention. At temperatures above 500° potassium and still more sodium iodide is appreciably volatile, and even at 500° small quantities of the order of 10γ volatilise very rapidly, though the loss is very much slower in the presence of alkali (see Fig. 1). Scheffer [1930] regards 500° as too high, but our results show that in the presence of sufficient alkali, heating can be carried out at 500° for prolonged periods without loss of iodine. Thus, in one experiment where 5γ iodine in the form of potassium iodide were added to blood together with 2 cc. of 80 % potassium carbonate, 5γ were recovered even after 2 hours' heating at 500°.

The blood was ashed by adding 1·5 cc. of approximately 10*N* potassium hydroxide to 10 cc. of blood in a nickel basin, drying thoroughly in an electric oven at 150°, and then heating at 500° for 1 hour in an electric muffle furnace

which had been calibrated by means of a pyrometer. If the preliminary drying at 150° is omitted, and the blood merely evaporated to dryness on a water-bath, there is great risk of loss through sputtering.

Extraction of iodide. The separation of the iodide from the other components of the ash is carried out as follows. The ash is very thoroughly extracted 4 times, each time with 4 cc. of distilled water, and the extracts filtered through a 3 G 4 Jena glass filter. The combined aqueous extracts are saturated with CO_2 to convert the hydroxide into the carbonate, and evaporated to dryness on the water-bath in a platinum or nickel basin. The carbonate

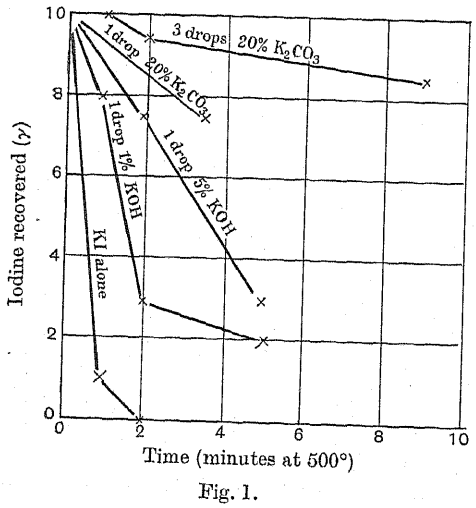


Fig. 1.

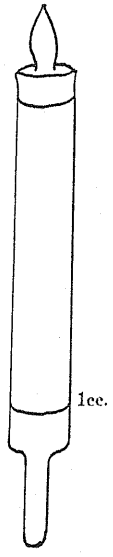


Fig. 2.

Fig. 1. 1 cc. of a solution containing 10γ iodine in the form of potassium iodide was placed in a platinum dish, and varying amounts of alkali added, as indicated on the curves. The diagram shows the amount of iodine recovered after heating in a muffle furnace at 500° for varying lengths of time.

residue is then extracted 4 times with 3 cc. of alcohol, the first extraction being carried out with 97 %, the second with absolute, and the last two again with 97 %. By this means, the carbonate becomes pasty, then rather granular, and finally quite liquid. The alcoholic extracts are carefully decanted from the carbonate residue and again filtered through a Jena glass filter. The combined alcoholic extracts, which contain all the iodide and very little of the other salts, are very carefully evaporated to dryness on the water-bath in a small platinum basin, the greatest care being taken, by covering the basin with an inverted funnel, to avoid any possibility of dust falling in, as this interferes seriously with the final colorimetric determination.

Liberation and extraction of iodine. The basin with its contents is then heated for 2 mins. in the muffle furnace at 500° to drive off traces of solvent

and any other organic matter, and the contents extracted repeatedly with small volumes of water to a total volume of 1 cc., the aqueous solution being poured off into one of the special stoppered tubes with a graduation at 1 cc. (Fig. 2). The iodine is then liberated by adding 1 drop of nitrosulphonic acid from a very fine pipette, and extracted by shaking very thoroughly with 0·05 cc. of pure carbon disulphide.

The use of carbon disulphide for this purpose is preferable to that of chloroform or carbon tetrachloride, as the mean partition coefficient for iodine between CS_2 and water is about 620, for $CHCl_3$ and water 130, and CCl_4 and water 87. The carbon disulphide was purified by adding about 3 % of pure iodine to remove iodine-binding substances, the iodine being removed next day by shaking with sodium hydroxide, and the carbon disulphide washed with water, dried over calcium chloride and distilled. It should be kept in a dark cupboard when not in use.

Colorimetric comparison. The tube is then compared with a set of standards freshly prepared by treating appropriate quantities of a standard potassium iodide solution with nitrosulphonic acid and carbon disulphide in an identical manner. The comparison should be carried out by daylight, or by the use of a daylight lamp.

A blank determination with the reagents used must always be carried out and the result subtracted from the result found. A fresh blank must be determined whenever any new reagent is taken into use.

Using this method it was found possible to recover quantitatively potassium iodide added to blood, and *p*-iodobenzoic acid was oxidised satisfactorily as is shown by the recovery of 2·0, 3·7 and 5·6γ of iodine when amounts of *p*-iodobenzoic acid containing 1·85, 3·7 and 5·6γ of iodine were added to blood.

The difficulty found in detecting small differences, in a total quantity of the order of 1γ (and this is the type of result to be expected working with 10 cc. of blood with an iodine content of about 10γ per 100 cc.) led us to try to produce a satisfactory titration method, where it might be expected that such quantities could be more accurately estimated.

Titration of small quantities of iodine, using starch as indicator.

Sensitivity of starch-iodine reaction. In attempting to apply this reaction to the determination of very small quantities, the first point requiring consideration is the sensitivity of the starch-iodine reaction, *i.e.* the visibility of the blue colour produced by small amounts of iodine in the presence of starch.

Table I shows our findings when various amounts of iodine in the form of potassium iodate were treated with varying amounts of iodide, 0·2 cc. $N/5$ sulphuric acid and 1 drop of 1 % soluble starch in small tubes, the total volume in each case being made up to 1·5 cc. with distilled water.

From these figures it is seen that something of the order of 0·08γ of "iodate-iodine" can be detected by this reaction. It might therefore be expected that titration results would be more or less consistently 0·1γ low on

Table I.

I as KIO_3 (γ)	KI			Free I present (γ)
	1000γ	2000γ	5000γ	
0·05	0	0	0	0·30
0·07	0	?	Faint trace	0·42
0·09	Faint trace	Faint trace	Trace	0·54
0·11	Trace	Trace	Pale blue	0·66

this account, but this was not found to occur, the error being at any rate roughly neutralised by the inevitable over-titration. Further, the KOH and K_2CO_3 used were not absolutely iodine-free, but had a blank of about 0·3γ in the quantities used for a determination, and even if one is not titrating to a correct end-point this blank correction is a constant which includes the titration error, and the latter will not therefore interfere with obtaining a true value for the unknown iodine.

Effect of iodide and acid concentration. The next point requiring consideration, and one which has been neglected by many workers, who add "a small crystal" of potassium iodide, is the effect of potassium iodide and acid concentrations on the titration value.

Our findings for the effects of potassium iodide concentration are given in Table II. The results were obtained by titrating a known amount of iodate-iodine (2γ) in presence of varying amounts of iodide with N/1000 thiosulphate from a microburette constructed from capillary tubing, with a delivery of 1·2 mm.^{3} per mm. of scale.

Table II.

Exp.	I as KIO_3 (γ)	I as KI (γ)	I found (γ)
1	2	20	1·31
2	2	50	1·4
3	2	100	1·6
4	2	200	1·6
5	2	500	1·73
6	2	1,000	2·02
7	2	2,000	1·98
8	2	5,000	1·93
9	2	10,000	2·02
10	2	20,000	2·08
11	2	50,000	2·85
12	2	100,000	4·73

In each experiment 2γ I (as KIO_3), the varying amounts of KI, and sufficient water to make a volume of 1·5 cc. were added to small titration vessels. 0·2 cc. N/10 H_2SO_4 and 1 drop of 1 % soluble starch were added, and the iodine liberated was then titrated with N/1000 thiosulphate which had immediately previously been standardised by macro-titration against potassium iodate.

From these results it is seen that reasonably accurate figures are obtained with a KI content between 1000γ and 2000γ, smaller quantities giving low results, and larger very high results. The end-point with the larger quantities of iodide is very unsatisfactory, the starch-iodine complex being of a dirty purple colour instead of a clear blue. If such a purple solution is allowed to stand, the complex separates as a deep purple precipitate with a colourless supernatant fluid.

It was found that substantially correct titration results were obtained when the acidity of the solution varied between 0·002 N and 0·05 N, but that lower concentrations of acid led to somewhat low results. Reith [1929] has found that correct results were obtained when the p_H of the solution varied between 1 and 6. If too strong acid is used, iodine may be liberated from KI even in the absence of iodate. Thus a mixture of 0·65 cc. water, 0·2 cc. 1 % KI, and 1·10 cc. of 10 % H_2SO_4, became blue immediately on adding starch. The concentration in this mixture is 1000γ KI per cc., and the acidity roughly N.

We can therefore, by choice of suitable conditions, titrate 2γ of iodate-iodine in a volume of 1·5 cc. with an error of, at most, $\pm$ 4 %.

Oxidation of HI to HIO_3. For this oxidation, chlorine water was first tried, as recommended by Veil and Sturm [1925], but as shown by various workers, especially Höjer [1928], even fresh chlorine water is unreliable and tends to give high results. Our work confirming this unsatisfactoriness will not be detailed.

Bromine water is much more satisfactory, and provided careful attention is paid to its freshness, quantitative oxidation of HI to HIO_3 can be carried out.

5γ iodine (as KI) in 1 cc. and 2 drops of $N/5$ H_2SO_4 were treated in a small pyrex tube, $2\frac{1}{2}'' \times \frac{3}{4}''$, through the bottom of which a piece of platinum wire had been fused to prevent bumping[1], with 3 drops of fresh bromine water. The tubes were then heated in a deep sand-bath and boiled vigorously for varying lengths of time. They were then cooled in ice, 2000γ KI (0·2 cc. 1 % KI) and 1 drop of 1 % soluble starch[2] added, and the contents titrated as before. It was found that satisfactory results were obtained when the solution was boiled for periods between 1 and 2 mins. Shorter boiling failed to remove all the bromine, and boiling for longer periods led to loss of iodine. If the solution were evaporated to dryness, no iodic acid at all was recovered.

A consecutive series of results obtained by this method, varying quantities of iodine being used, and a constant boiling time of 1 min. being employed, is given below (Table III). With the exception of No. 17, these results can be claimed as satisfactory, at any rate for quantities greater than 0·1γ.

Efficiency of extraction of carbonate residue. To test the efficiency of the extraction of the iodide from the carbonate residue, 10γ iodine (as KI) were added to 2 cc. of 80 % potassium carbonate in an 8 cm. nickel basin and evaporated to dryness in an air-oven at 150°. The dry carbonate was extracted four times with 3 cc. of alcohol, by grinding up very thoroughly with a nickel spatula, and decanting the separate alcoholic extracts into the small titration vessel. The alcohol was then evaporated off on the sand-bath and the dry residue heated $1\frac{1}{2}$ min. on the sand-bath to drive off the last traces of alcohol.

[1] We were using this device for some months before the publication of Aitken [1930] on the determination of iodine.

[2] The starch should not be too old, but we found it to keep unimpaired in the ice-chest for at least a week.

Table III.

Exp.	I as KI oxidised (γ)	I as HIO_3 found (γ)	Exp.	I as KI oxidised (γ)	I as HIO_3 found (γ)
1	5·0	5·07	11	1·0	1·01
2	,,	5·07	12	0·5	0·55
3	,,	5·12	13	,,	0·52
4	,,	5·07	14	,,	0·52
5	2·5	2·58	15	0·25	0·25
6	,,	2·49	16	,,	0·25
7	,,	2·49	17	,,	0·42
8	,,	2·56	18	,,	0·22
9	1·0	1·04	19	0·10	0·08
10	,,	0·96	20	,,	0·06

The iodine content of each fraction was determined as follows. The residue was dissolved in 1 cc. water, neutralised to Congo red (as external indicator) with $N/5$ H_2SO_4, 2 drops of acid were added in excess and the oxidation with bromine was carried out as previously described. Two such experiments were carried out, one with absolute, the other with about 90 % alcohol.

Table IV.

	Absolute alcohol		90 % alcohol	
Solvent	I found (γ)	% of total I	I found (γ)	% of Total I
First extraction	4·4	43	8·4	81
Second ,,	2·0	19	1·4	14
Third ,,	3·2*	31	0·9	9
Fourth ,,	0·9	9	0·2	2
Total	10·5		10·9	

(Total I content, including K_2CO_3 blank, 10·3γ.)

* Sufficient water was added here to make the carbonate layer a liquid paste.

The figures (Table IV) clearly show that the extraction with the more dilute alcohol is very much more rapid and complete than that with the absolute, in accord with the findings of Scheffer [1930], and, in practice, the rather liquid residue is more easy to deal with than the sticky solid obtained with the absolute alcohol. The only disadvantage is that rather more alkali is taken up by the weaker alcohol. In subsequent work, absolute and 90 % alcohol were used as required to keep the carbonate pasty, without becoming either granular or liquid.

To avoid the necessity of filtering and carbonating the aqueous extract of the ash, as described in the colorimetric section, it seemed desirable to try ashing the blood with carbonate instead of potassium hydroxide, and extracting the ash after ignition for 1 hour at 500° directly with four quantities of 3 cc. alcohol. The alcoholic extracts were then evaporated to dryness in the water-bath in 5 cm. nickel basins, and the dry residue again extracted with alcohol, 1 cc. followed by three amounts of 0·5 cc. being used. The extracts were poured into the little glass vessels, evaporated to dryness on the sand-bath, heated a further 90 secs. and acidified and oxidised with bromine as before.

The results of experiments on mixtures of potassium carbonate and iodide (without blood) showed that the whole process, including the two separate alcoholic extractions could be carried out satisfactorily, the recoveries in four successive experiments with 2·5γ iodine as KI being 2·46, 2·46, 2·76, 2·66γ, and potassium iodide added to blood could also be recovered fairly satisfactorily, though results were sometimes a little low. It was found that when the blood was not carefully burned, and a large amount of carbon residue was left, direct alcohol extraction failed to remove all the iodine, though that missing could be recovered by re-ashing the residue and again extracting, or better by a first aqueous extraction. Unfortunately, when the whole procedure was carried out, using carbonate as the alkali for ashing, and thyroxine or *p*-iodobenzoic acid were added to blood, the recoveries were usually low, values of the order of 85–95 % of the added iodine being recovered. When the work had reached this stage further experiments had, unfortunately, to be abandoned. It would therefore seem that under these conditions, potassium carbonate is not entirely satisfactory as an alkali for ashing the blood, though further work might enable suitable conditions to be worked out.

It is suggested that a suitable method would probably be found by ashing the blood with potassium hydroxide as described in the colorimetric method, filtering and carbonating the aqueous extract of the ash, and then working up the dried carbonate containing the iodine by two alcoholic extractions and bromine oxidation as described above.

Our thanks are due to Prof. F. R. Fraser for the interest he has shown throughout.

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(The above papers contain fairly complete references to the extensive literature of the subject.)

CXCIX. DEHYDROGENATIONS BY BRAIN TISSUE. THE EFFECTS OF NARCOTICS.

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(*Received August 27th, 1932.*)

It has recently been shown [Quastel and Wheatley, 1932, 2] that exposure of fresh brain tissue to narcotics at low concentrations results in specific inhibitory effects on the oxidations of the brain. The narcotics exert a profound inhibitory action on the oxidation of substances important in carbohydrate metabolism, *viz.* glucose and lactic and pyruvic acids, this action being accomplished at concentrations of narcotics which are of the same order as those required to produce deep narcosis in animals. The narcotics, however, at these concentrations, do not interfere with the oxidation by the brain of sodium succinate or *p*-phenylenediamine. This indicates that their behaviour is not due to a disturbance of the brain-cell whereby its mechanisms for accomplishing oxidations as a whole are affected. Their behaviour appears to be due to an effect, possibly at cell interfaces, whereby the mechanisms which result in an activation of the molecules of lactic and pyruvic acids are greatly disturbed. It has also been shown that among narcotics belonging to the same chemical type those with the greater hypnotic activity have the greater inhibitory action on the oxidation by brain of glucose or lactic acid. The conclusion was drawn that an adsorption of the narcotic took place at a specific area or centre of the nervous system. This resulted in an inhibition of the oxidation of glucose, and of lactic and pyruvic acids by the cells constituting this nervous centre. The access or activation of oxygen was unimpaired, but the diminished ability of the cells to oxidise glucose or lactic acid resulted in a lowering of the amount of energy available for the cells to accomplish their functional activities. Narcosis—or a depression of the normal functional activity of the nervous centre in question—might then ensue.

It follows, if this view is correct, that the action of the narcotic will be reflected not only in diminished oxygen uptake by lactic acid or glucose but in a diminished power of the brain-cell to effect the anaerobic oxidation of glucose or lactic acid, the fundamental phenomenon being a disturbance of the mechanism involving the activation of glucose, lactic acid or pyruvic acid.

This paper is concerned with the dehydrogenating powers of brain tissue and of the effects of a number of narcotics on these. It will be shown, in full

confirmation of earlier results, that narcotics at low concentration share the general property of markedly inhibiting dehydrogenations by the brain of glucose, lactic acid or pyruvic acid.

Technique.

Brain tissue was obtained as soon as possible after death of the animal. In the case of large animals such as sheep or bullocks a maximum period of 3 hours elapsed between death of the animal and examination of the brain. Storage where circumstances made this necessary was always at 0°. A serious difficulty in working with human material was to obtain brain tissue less than 6 hours old; nevertheless results with human tissue have been such as to confirm fully results with the tissues of other animals, and no evidence has been secured as yet to indicate that human material differs more than quantitatively, so far as dehydrogenating mechanisms are concerned, from that of the animals studied in this investigation.

The *pia* was removed from the whole brain, which, after being freed from all membrane and possible blood clots, was well minced under ice-cold conditions and made into a homogeneous mixture by pressing through muslin. A portion was then mixed with three times its weight of saline, shaken in a machine and finally filtered through muslin. A creamy homogeneous suspension was obtained which proved to be satisfactory for investigations of dehydrogenating power. The suspension must be kept at 0° and used as freshly as possible. The effect of age on the activity of the suspension will be discussed later.

Grey and white matter have been compared, using mostly human material for this purpose. Suspensions of grey and white matter were made up in saline as indicated for whole brain.

Dehydrogenase activity was investigated by the Thunberg methylene blue technique. Each vacuum tube contained 2 cc. $M/15$ phosphate buffer solution p_H 7·4, 2 cc. of the suspension prepared as above, 1 cc. 1/5000 methylene blue solution and 1 cc. of a solution containing the substance under investigation. The tube was evacuated and the reduction time of the methylene blue at 37° was noted. Whenever a suspension gave a reduction time, in the absence of added hydrogen donators, of less than 20 minutes, it was diluted with saline until the suspension gave a reduction time of approximately 1 hour.

Dehydrogenations by the brain.

Thunberg [1923] has already shown that nervous tissue possesses the power of activating a number of substrates as hydrogen donators. Such substances are glucose, sodium lactate, sodium succinate, sodium citrate. Ashford and Holmes [1931] have stated that lactate, succinate, glycerophosphate and glucose have approximately the same activity. Actually, although these sub-

stances are, unquestionably, active donators of hydrogen in presence of brain tissue, there are considerable differences between them so far as their activities are concerned.

A point of some importance in carrying out this work is the effect of relatively high concentrations of salt upon brain dehydrogenations. If a suspension of brain tissue be made up in distilled water, it has relatively little reducing power when examined under the conditions specified above. If, however, physiological saline instead of water be added to the tissue there is a marked increase in reducing power. In a typical experiment a reduction time of 120 mins. was changed to 14 mins. It seems that the change in osmotic concentration in the solution surrounding the brain-cells results in the withdrawal from the tissue of certain substances which effect the reduction of methylene blue adsorbed at the cell surfaces, or possibly the change of salt concentration affects the permeability of brain-cells towards methylene blue. In view of this result it has been necessary in studying dehydrogenating action to use tissue and substrates made up in saline solution and to carry out adequate controls in order to form an estimate of the effects of changes in osmotic concentration.

Table I gives a typical series of results obtained with bullock (whole) brain.

Table I. *Reduction times due to various substrates in presence of bullock (whole) brain.*

Substrate	Reduction time (in minutes)
Saline (physiological)	100
Saline (0·2 *M*)	51
Glucose (0·16 *M*)	25
Fructose (0·16 *M*)	40
Galactose (0·16 *M*)	22
Mannitol (0·16 *M*)	27
Sodium lactate (0·13 *M*)	14
Sodium citrate (0·03 *M*)	40
Sodium glutamate (0·13 *M*)	28
Sodium succinate (0·13 *M*)	21
Sodium glycerophosphate (0·13 *M*)	16

(*a*) *The sugars and polyhydric alcohols.* Glucose, fructose, galactose and mannitol, at the concentrations quoted, are all activated as hydrogen donators. It is easy to explain the reducing activity of glucose on the basis of rapid glycolysis to lactic acid which is an efficient hydrogen donator with brain. It is much more difficult to explain the activities of the other sugars on these lines. Löbel [1925] states that fructose gives rise to little or no lactic acid in the presence of brain, yet it is oxidised freely, and its oxidation, like that of glucose, is inhibited by preliminary treatment of brain tissue with very dilute iodoacetic acid [Quastel and Wheatley, 1932, 1]. Fructose, therefore, is either oxidised by brain in a different manner from glucose (whose oxidation is known to proceed largely *via* lactic acid) or it gives rise to a small quantity of lactic acid just sufficient to account for its oxidation by oxygen or methylene blue. Mannitol and galactose, however, which are

hydrogen donators, do not take up oxygen in presence of brain at an appreciable rate [Quastel and Wheatley, 1932, 1]. Presumably these substances are sufficiently activated by the brain to reduce methylene blue, but insufficiently so to reduce at any marked rate the natural respiratory carrier (? cytochrome) of the brain.

(*b*) *Succinic, lactic, pyruvic, citric, glutamic and glycerophosphoric acids.* These substances, used as their sodium salts, reduce methylene blue in presence of brain tissue. They are all capable, too, of being oxidised by this tissue, their presence resulting in a marked oxygen uptake by brain [Quastel and Wheatley, 1931; 1932, 1; Ashford and Holmes, 1931].

The activities of a number of these substances, as well as those of glucose and fructose, are shown in Figs. 1 and 2 where the velocity of reduction is plotted against the logarithm of the concentration.

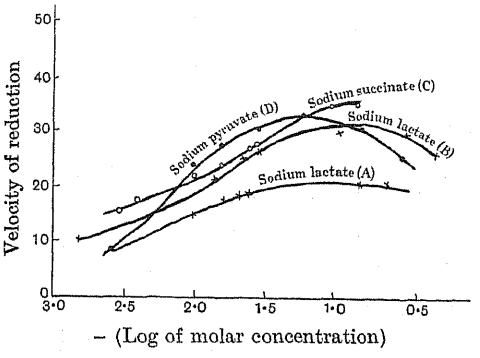


Fig. 1.

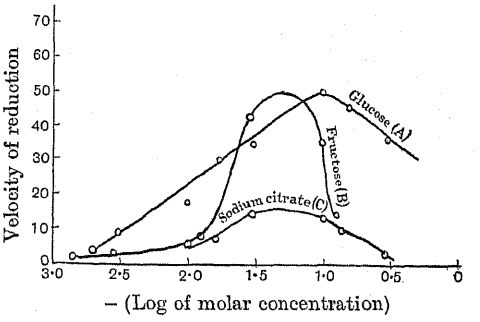


Fig. 2.

The velocity of reduction of the hydrogen donator has been calculated on the basis that the observed velocity of reduction is the sum of that due to the added donator and that of the tissue itself. This must be considered correct only to a first approximation.

It will be noted that velocity – log conc. curves show in general the same phenomenon: an increase in velocity with concentration to a maximum after which there is a decrease in velocity as the concentration is increased. It is important therefore in estimating relative activities of hydrogen donators to determine the rates of reduction with varying concentrations of the substrates.

Such a general phenomenon probably has a common explanation in all cases. It is similar to that described by Dixon and Thurlow [1924], who, working with the xanthine oxidase of milk, showed that increase of concentration of the hydrogen donator led, after the attainment of a peak, to a considerable drop in reduction velocity. This was ascribed to a blocking of the enzyme by excess hypoxanthine which in this way hindered the access of methylene blue and retarded the velocity of reduction. This view seems to be confirmed by the recent results of Harrison [1932]. It is probable that the

results incorporated in Figs. 1 and 2 have a similar explanation. Bernheim [1928] noted the same phenomenon in respect to the dehydrogenation of citric acid using an enzyme preparation from liver.

In Table II are shown the concentrations of a number of hydrogen donators at which half the maximum velocity of reduction in presence of brain tissue is attained. The very high activities of succinic, lactic and pyruvic acids compared with those of glucose and fructose are noteworthy.

Table II.

Substrate	Concentration at which half maximum velocity is reached (M)
Glucose	0·0135
Fructose	0·021
Sodium lactate	0·0045
Sodium pyruvate	0·0052
Sodium citrate	0·018
Sodium succinate	0·004

Comparison of the activities of grey and white matter.

A comparison of the dehydrogenating abilities of grey and white matter shows that the former is three to four times as active as the latter in respect of all hydrogen donators investigated. This refers to bullock, sheep and human brain. Table III gives a representative series of results. It is to be noted that this difference in activity is precisely that found between the rates of oxygen uptake of grey and white matter in the presence of various substrates [Quastel and Wheatley, 1932, 1].

Table III. *Reduction times in minutes of hydrogen donators in presence of human grey or white matter (equal weights).*

Substrate	Grey matter	White matter
Saline	65	120
Glucose (0·18 M)	18	54
Fructose (0·18 M)	25	75
Galactose (0·18 M)	15	60
Sodium glycerophosphate (0·13 M)	16	51
Sodium succinate (0·13 M)	9	27

Effects of storage at 0° on the lability of brain dehydrogenases.

Storage of a brain suspension at 0° results in a slow inactivation of the dehydrogenases. The activities due to the sugars disappear more quickly than those of succinic and glycerophosphoric acids. Table IV, which incorporates representative results, shows that the lability of the mechanisms concerned with the sugars is very considerable, that with mannitol being still more marked. Brain tissue which has not been made into a suspension but allowed to stand intact at 0° shows the same phenomenon, though the effects are slower than with a brain suspension. With intact brain tissue the velocity of reduction of methylene blue due to added glucose does not decrease at a rate

Table IV. *Effects of storage of brain suspension (bullock) at 0° on the activity of dehydrogenases.*

Reduction times in minutes.

Substrate	Period of storage at 0° (hrs.)		
	3	49	74
Saline	18	35·0	49
Glucose (0·18 *M*)	7·4	22	25
Fructose (0·18 *M*)	9·0	19	27
Galactose (0·18 *M*)	6·4	18	22
Mannitol (0·18 *M*)	8·6	27	46
Sodium succinate (0·13 *M*)	6·0	13	14
Sodium glycerophosphate (0·13 *M*)	6·8	12	14
Sodium citrate (0·03 *M*)	7·0	24	24

linearly proportional to the time of storage, but remains fairly steady for 20 to 40 hours, after which there is a rapid decrease in the velocity of reduction, which becomes more marked as the time of storage is prolonged.

There is some evidence to show that if a tissue suspension be kept anaerobically (in nitrogen or hydrogen) at 0° the dehydrogenases retain their activity for a longer period than when the tissue is stored at 0° in the presence of oxygen (see Table V).

Table V. *Effects of storage of brain suspensions (sheep) for 18 hours at 0° in oxygen and in hydrogen.*

Reduction time in minutes.

Substrate	Storage in oxygen	Storage in hydrogen
Saline	>100	>100
Glucose (0·18 *M*)	>100	33
Mannitol (0·18 *M*)	>100	31

Lability of brain dehydrogenases at 37°.

The activities of a brain tissue suspension towards the sugars fall off very rapidly on prolonged incubation of the suspension at 37°. The citric acid dehydrogenase is also very labile whilst those of succinic, lactic and glycerophosphoric acids are relatively more stable (see Table VI).

Table VI. *Effect of incubation of brain tissue (sheep) suspensions at 37° on dehydrogenations.*

Reduction times in minutes.

Substrate	Time of incubation at 37° (mins.)	
	0	60
Glucose (0·16 *M*)	56	110
Fructose (0·16 *M*)	40	84
Sodium succinate (0·12 *M*)	22	25
Sodium lactate (0·6 *M*)	8	10
Sodium citrate (0·03 *M*)	6	14
Sodium glycerophosphate (0·13 *M*)	12	12

These facts are of importance in investigating the action of narcotics and it is essential to realise when considering the effects of inhibitors on brain dehydrogenases that prolonged exposure at 37° under our experimental conditions is in itself detrimental to some of the mechanisms involved in bringing about dehydrogenations. Doubtless this phenomenon is responsible to some extent for the relatively high values found for the concentrations of sugars which are necessary to achieve half the maximum velocity (Table II). It cannot account entirely for the high values found, owing to the fact that the reduction times obtained with the sugars (used in compiling Table II) were smaller than those required to effect any appreciable destruction of the relevant dehydrogenases by incubation at 37° alone.

The action of somnifaine on brain dehydrogenations.

Somnifaine[1] is a drug composed of equal parts of veronal and allyl*iso*propylbarbituric acid and is commonly used in narcosis treatment of psychotic subjects. It has been shown [Quastel and Wheatley, 1932, 2] that exposure of brain tissue to the drug results in a considerable lowering in the rate of oxygen uptake, this amounting to a decrease of more than 50 % in the velocity of oxidation and taking place with concentrations of the narcotic which are of the same order as those required to induce narcosis. Moreover, the inhibitory action of the drug was found to be restricted to the oxidations of glucose, lactic and pyruvic acids.

Exposure of brain tissue to somnifaine at a concentration of 0·4 % resulted in a complete inhibition of the tissue's own reducing ability and of the power of the tissue to activate the sugars. Using a concentration of 0·1 % there is a marked increase in the reduction time of the tissue alone, and the power to activate glucose is reduced by 50 %. The activations, however, of succinic acid and glycerophosphoric acid are much less disturbed. Representative results are shown in Table VII.

Table VII. *Reduction times in minutes of various substrates in the presence of brain tissue (bullock) alone and together with somnifaine (0·1 %).*

Both the tissue alone and the tissue and somnifaine were incubated for 30 mins. at 37° prior to addition of substrate and methylene blue solution.

Substrate	Reduction time alone	Reduction time in presence of somnifaine
Glucose (0·16 *M*)	23	54
Fructose (0·16 *M*)	26	74
Galactose (0·16 *M*)	37	>100
Mannitol (0·16 *M*)	30	95
Sodium succinate (0·11 *M*)	21	26
Sodium glycerophosphate (0·11 *M*)	12	14

[1] Somnifaine, as used for injection purposes, is an alcohol-glycerol-water solution of a mixture of 10 % veronal and 10 % allyl*iso*propylbarbituric acid.

These results fully confirm earlier work [Quastel and Wheatley, 1932, 2] and show the specificity of inhibition evinced by the narcotic towards the activation of glucose.

Effects of incubating brain tissue with somnifaine.

If a brain suspension be incubated with somnifaine (0·1 %) for various periods at 37° it is found that there is progressively more and more inhibition of glucose activation as the time of incubation increases, the inhibitions being much greater than in the absence of the narcotic. Representative results are noted graphically in Fig. 3, where it will be seen that whereas the glucose activation decreases with length of incubation of the brain tissue with somnifaine, that of succinic acid or glycerophosphoric acid is but little affected.

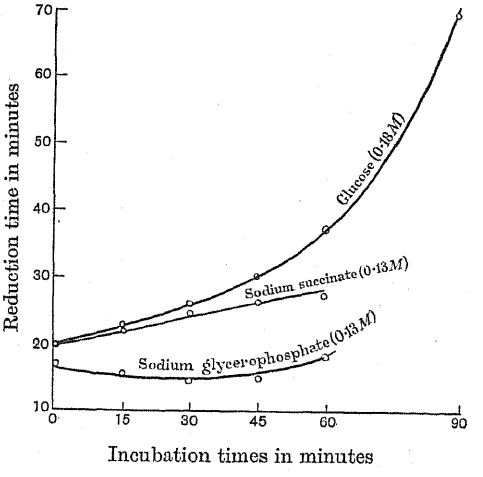


Fig. 3. Effects of incubation of brain tissue with somnifaine (0·1 %) on reduction times of substrates.

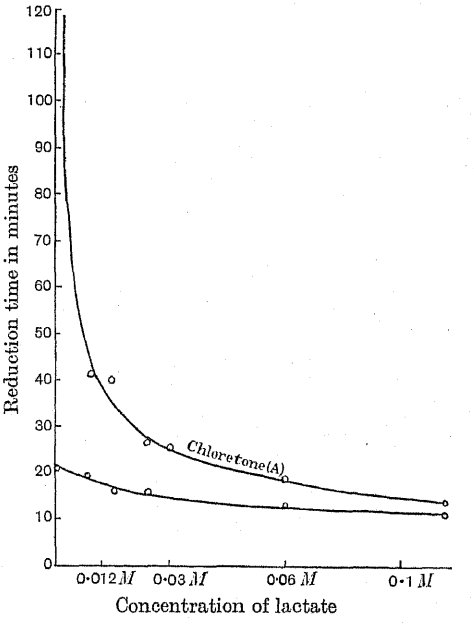


Fig. 4.

The activations of fructose, mannitol and galactose are influenced in a similar manner to that of glucose. These effects have been found to occur with the brains of guinea-pig, rabbit, sheep, bullock and man.

The action of luminal and veronal on brain dehydrogenations.

Luminal, phenylethylbarbituric acid, behaves qualitatively in precisely the same way as somnifaine so far as brain dehydrogenations are affected. Veronal, which has much less hypnotic power than luminal or somnifaine, has much less inhibiting activity. These facts are in accordance with those

found in the study of the effects of narcotics on the oxygen uptake of brain. There exists a definite parallelism between hypnotic activity of the narcotic and its power to inhibit the oxidation by brain of glucose, lactic acid or pyruvic acid [Quastel and Wheatley, 1932, 2].

Results illustrating the effects of luminal and veronal on the activity of brain tissue towards the sugars are shown in Table VIII. These narcotics have little or no action on the dehydrogenation of succinic and glycerophosphoric acids.

Table VIII. *Effects of incubating brain tissue (sheep) for 30 mins. at 37° alone and in the presence of luminal (0·2 %) and veronal (0·2 %) on the activity of the dehydrogenases.*

Reduction times in minutes.

Substrate	Tissue alone	Tissue with luminal	Tissue with veronal
Glucose (0·16 *M*)	22	51	26
Fructose (0·16 *M*)	22	54	26
Galactose (0·16 *M*)	22	78	26
Mannitol (0·16 *M*)	32	78	40
Sodium succinate (0·11 *M*)	24	33	25
Sodium glycerophosphate (0·11 *M*)	24	24	24

The effect of luminal on the activation of lactic acid.

Neither somnifaine (0·1 %) nor luminal (0·2 %) appears to have an appreciable retarding effect on the reduction of methylene blue by brain tissue in presence of high concentrations of sodium lactate. In fact somnifaine seems to have as little effect on the activation of 0·3 *M* or 0·5 *M* sodium lactate as upon the activation of sodium succinate. When however the lactic acid concentration is decreased to limits which are those found in brain tissue itself somnifaine and luminal effect very considerable inactivations. Typical results with luminal are noted in Table IX.

Table IX. *Reduction times in minutes of varying concentrations of sodium lactate in presence of brain tissue (bullock) alone and with luminal (0·2 %).*

The saline controls gave reduction times >100 minutes.

Sodium lactate conc. (*M*)	Tissue alone	Tissue with luminal
0·03	39	37
0·024	40	47
0·015	45	73
0·003	90	>100

Luminal also brings about an elimination of the activation of sodium pyruvate when this is present at low concentrations (a normal reduction time of 51 mins. was increased to 144 mins. using pyruvate at a concentration of 0·003 *M*). This inhibiting effect of luminal (0·2 %) on the activation of lactate or pyruvate at low concentration does not take place to the same marked extent with low concentration of succinate or glycerophosphate. Here reduction times were increased (at 0·003 *M* concentration) from 15 to 16 mins. in

the case of succinate and were unchanged in the case of glycerophosphate (26 mins.).

It appears that the narcotic (somnifaine or luminal) is only effective as an inhibitor of lactic acid activation when this is present at low concentrations. This fact is of some importance as it shows that the narcotic does not inactivate a portion of the enzyme irreversibly; if this occurred the reduction times of high concentrations of lactate would be affected to the same extent as those of low. The facts indicate that the inhibiting action of the narcotic is inversely proportional to the concentration of lactic acid present and point to a competition between the drug and the lactic acid for the dehydrogenase concerned.

The action of chloretone.

Chloretone, a hypnotic of widely different chemical constitution from the dialkylbarbituric acid series, shows the same qualitative effects as somnifaine or luminal on brain dehydrogenases. Its action on the activation of lactic acid at varying concentrations of this donator is shown in Fig. 4 (p. 1679). It is found that the curve (curve *A*, Fig. 4) representing the action of chloretone inhibition on lactic acid oxidation is identical with that mathematically derived on the assumption that chloretone and lactic acid compete for the same activating centre or dehydrogenase.

The equations involved are

$$\underset{\text{(Enzyme)}}{E} + \underset{\text{(Chloretone)}}{C} \rightleftarrows EC$$

$$E + \underset{\text{(Lactic acid)}}{L} \rightleftarrows EL$$

$$EL \rightarrow E + \text{oxidation product of lactic acid.}$$

Let e be the total molar concentration of enzyme,
c ,, ,, chloretone,
l ,, ,, lactic acid.

Let K_l = dissociation constant of EL and K_c = dissociation constant of EC. Let x = the concentration of EL molecules and y = the concentration of EC molecules. Then the concentration of E molecules is $(e - x - y)$.

We have
$$K_l x = l\,(e - x - y) \qquad \text{.....(i),}$$
$$K_c y = c\,(e - x - y) \qquad \text{.....(ii),}$$

e being small compared with c or l.

Then we have [*cf.* Haldane, 1930]

$$\frac{v'}{v} = \frac{1}{1 + \frac{K_l c}{K_c(K_l + l)}},$$

where v' = velocity of reduction in presence of chloretone (for constant quantity of methylene blue)

and v = velocity of reduction in absence of chloretone.

Table X gives reduction times calculated from the above equation taking

$K_l = 0{\cdot}0045$ (Michaelis constant),
$K_c = 0{\cdot}0038$.

K_c was calculated from the following pair of points:

reduction time in presence of chloretone = 25 mins.
" absence " = 14 mins.

K_l, the Michaelis constant, was derived from an entirely different set of readings (see Table II).

It will be noted that there is a striking agreement between the calculated and observed results for the reduction times in presence of chloretone. The starred figure was taken from the curve Fig. 4. The results confirm the hypothesis that a competition exists between chloretone and sodium lactate for the dehydrogenase. Similar results were obtained with luminal.

Table X. *Reduction times in minutes of sheep brain tissue in presence of sodium lactate with and without chloretone (0·023 M).*

Controls with saline gave reduction times >120 minutes.

Molar concentration of sodium lactate	Reduction time in absence of chloretone	Reduction time with chloretone (observed)	Reduction time with chloretone (calculated)
0·12	11	14	13·4
0·06	13	18	18
0·03	14	25	25
0·024	15	26	29
0·015	18	41	43
0·003	21	100*	97

The concentration of lactate required to give half the maximum velocity of reduction is raised from 0·0045 *M* to 0·065 *M* by subjection of brain (bullock) tissue to 0·4 % chloretone.

Reversibility of narcotic action.

On adding a mixture of glucose and somnifaine (or luminal) to brain tissue and incubating this for varying intervals of time at 37°, reduction times of methylene blue added at the end of these periods are much smaller than those obtained if glucose is added after the tissue has been incubated in the presence of the narcotic. The addition of glucose at the same time as the narcotic results apparently in a protective action. The following are typical results:

Reduction time by brain tissue (bullock) incubated with somnifaine (0·1 %) and glucose (0·18 *M*) for 30 mins. ... 12 mins.

Reduction time by the same tissue incubated with somnifaine (0·1 %) for 30 mins. glucose (0·18 *M*) then being added 24 mins.

Löbel [1925] has shown that narcotics at low concentrations do not interfere greatly with glycolysis by brain and it would seem, in view of the well-known

high glycolytic power of brain tissue, that the protective action described is simply due to lactic acid being produced from the glucose at a concentration sufficiently high to overcome the inhibitory action of the narcotic. This idea seems reasonable in view of the facts already given indicating the existence of competition between the narcotic and lactic acid for the dehydrogenase concerned. On the other hand there still remains the possibility that a true protection against the narcotic is afforded by glucose itself. In support of this there is evidence that both fructose and galactose show "protective" effects against somnifaine which are of the same order as that demonstrated by glucose. It is therefore clear that the problem is not yet settled and must remain in abeyance until more is known of the details of metabolism of the various sugars in presence of brain.

SUMMARY.

1. The dehydrogenating powers of brain tissue of various animals have been examined and the Michaelis constants have been determined for a number of substrates. Determinations of velocity-concentration curves have been made. The curves all exhibit maxima, above which increase of concentration leads to decreased reduction velocities.

2. Grey matter is three to four times as active as white matter.

3. Glucose, fructose, galactose and mannitol are activated as hydrogen donators by fresh brain tissue. The activities of glucose and fructose are less than those of sodium succinate, sodium lactate and sodium pyruvate.

4. The activations of the sugars disappear more quickly on storage of the brain at 0° or on incubation at 37° than those of sodium succinate and glycerophosphate.

5. Exposure of brain tissue to narcotics (somnifaine, luminal, chloretone) results in an elimination of the activation of the sugars, those of sodium succinate and glycerophosphate being relatively undisturbed.

6. The activations of sodium lactate and sodium pyruvate at low concentration are inhibited by the narcotics.

7. It is shown that the narcotic and sodium lactate compete for the same activating enzyme or centre. The narcotic does not inactivate the lactic dehydrogenase irreversibly but its inhibiting action is dependent on the concentration of lactic acid present.

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CC. THE ACTION OF DYESTUFFS ON ENZYMES.

III. UREASE.

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(*Received August 31st, 1932.*)

The action of dyestuffs on bacterial dehydrogenases and on an intracellular enzyme, fumarase, has been discussed in earlier communications [Quastel and Wheatley, 1931; Quastel, 1931]. It is now proposed to describe the effects of dyestuffs on another widely distributed intracellular enzyme, urease.

Technique.

The method of investigation consisted in submitting a preparation of urease to the action of a dyestuff at p_H 7·4 and at 45° for a given time, usually 30 mins. or 1 hour, and in then determining the activity of the enzyme by estimating the amount of ammonia produced at 45° in a given time from a 0·3 % solution of urea. 1 cc. of the enzyme preparation was added to 2 cc. $M/15$ phosphate buffer solution p_H 7·4 and then the dyestuff and any other substance under investigation. The volume was made up to 9 cc. with distilled water and the mixture was incubated at 45° for the required interval of time (referred to as the initial incubation period). 1 cc. 3 % urea was then added and incubation was allowed to proceed at 45° for a further interval (referred to as the final incubation period). At the close of this interval the free ammonia was estimated on 1 cc. by a suitable aeration method and compared with that obtained from urease treated under similar conditions but in the absence of dyestuff. The amount of enzyme preparation taken was usually that required to decompose 1 cc. 3 % urea at 45° and at an initial p_H of 7·4 in one hour.

Effects of dyestuffs on soya bean urease and on a purified urease preparation.

A soya bean preparation of urease was made by shaking 10 g. soya bean meal with 90 cc. distilled water for 30 mins. in a shaking machine and centrifuging. The centrifugate was used as the enzyme preparation.

The purified urease preparation was made from a commercial sample of urease. The origin of the enzyme in this preparation was soya bean, purification having been effected by removal, to a considerable extent, of proteins by acid precipitation.

Representative results showing the effects of 23 dyestuffs, each at a concentration of 1/5000, on the activities of the two preparations of urease are shown in Table I. The percentage inhibitions of the activities of the enzymes are recorded.

Table I. *Percentage inhibitions of the activities of soya bean urease and a purified preparation of urease effected by dyestuffs (1/5000).*

Dyestuff	Nature of dyestuff	Soya bean preparation	Purified preparation
Congo red	Acid	0	0
Benzopurpurin	,,	0	0
Orange G	,,	0	0
Crystal scarlet	,,	0	0
Acid fuchsin	,,	0	0
Acid green	,,	0	0
Soluble blue	,,	0	0
Water blue	,,	0	0
Brilliant green	Basic	90	3
Malachite green	,,	95	86
Methyl violet	,,	96	62
Ethyl violet	,,	95	66
Crystal violet	,,	93	50
Methylene blue	,,	0	21
Toluidine blue	,,	0	28
Neutral red	,,	0	74
Janus green	,,	5	53
Safranine	,,	0	18
Methylene violet	,,	0	10
Bismarck brown	,,	0	0
Auramine O	,,	0	0
Pyronin	,,	0	0
Eosin	,,	0	0

The following facts are of note.

1. The acid dyestuffs are entirely inert. This result may be contrasted with that found in the investigation of the action of dyestuffs on fumarase. Here Congo red, for instance, was toxic at a concentration of 1/100,000 [Quastel, 1931].

2. Most basic dyestuffs are toxic. The toxicity depends greatly on the nature of the preparation of the enzyme.

Basic dyes of the triphenylmethane series (brilliant green, malachite green, methyl violet, ethyl violet, crystal violet) show over 90 % inhibition of the activity of a soya bean preparation of urease. Yet this series proves to be less toxic with the purified preparation of the enzyme. Brilliant green, indeed, is almost inert with the purified enzyme at the concentration quoted. This finding with the triphenylmethane series may be contrasted with that obtained with neutral red or Janus green. These two dyes are highly toxic to the purified enzyme but they have no toxicity at the given concentration to the soya bean preparation. The same may be said of methylene blue or toluidine blue which have relatively feeble toxicities.

It was found from earlier work that the presence of proteins protected enzymes from dyestuffs (including the triphenylmethane series). The proteins combined with the dyes rendering them inert. This has been found to be the

case with neutral red, Janus green, *etc.*, no toxicity to urease being demonstrated using a soya bean preparation which contains much protein. It was very surprising therefore to find that the basic triphenylmethane series of dyes apparently lose their toxicity on purification of the enzyme preparation.

When greater concentrations of this series of dyes are used their toxicity towards the purified enzyme becomes evident. This is shown in Table II. It is clear that the triphenylmethane series of dyes possesses some property, not possessed by other basic dyes, which renders them more toxic to the soya bean preparation of urease than to the enzyme in the purified condition.

Table II. *Percentage inhibition of the activities of a soya bean preparation and a purified preparation of urease effected by varying concentrations of dyestuffs.*

	Concentration of dye		
Dyestuff	1/2000	1/3300	1/10,000
Purified urease preparation:			
Brilliant green	92	71	3
Methyl violet	92	88	35
Congo red	0	0	0
Neutral red	88	81	51
Acriflavine	72	69	3
Safranine	59	32	0
Methylene violet	54	19	0
Methylene blue	69	42	30
Bismarck brown	19	14	0
Orange G	0	0	0
Eosin	0	0	0
Soya bean preparation:			
Brilliant green	92	91	91
Methyl violet	94	94	93
Congo red	0	0	0
Neutral red	0	0	0
Acriflavine	46	37	6
Safranine	0	0	0
Methylene blue	0	0	0
Trypan blue	0	0	0

The relative inertness of these dyes to the purified enzyme is shown also to a preparation of urease obtained from soya bean by Zakowski's method [1931] in which purification is effected by precipitation of proteins *etc.* by CO_2. If urease, purified in this way, is neutralised and examined in the usual manner, it is found that brilliant green at a concentration of 1/50,000 has lost its toxic activity. The addition to the purified preparation of the enzyme of a little of the material precipitated from soya bean by CO_2, however, restores the toxicity of the brilliant green. The dye now effects a 90 % inhibition of activity. There is thus a co-toxic factor in the soya bean which is removed by acid or CO_2 precipitation. This phenomenon will be discussed in more detail later.

The inertness of all the acid dyestuffs investigated was to some extent unexpected. Urease has, according to Lövgren [1921] an optimum p_H between 7 and 8, which varies according to the substrate concentration. It

would have been supposed that the enzyme would have ampholytic properties and combine, as fumarase does, with both acidic and basic dyestuffs. The fact that the acidic dyestuffs investigated are inert would mean either (1) that an insufficient range of acid dyestuffs has been investigated, or (2) that it is incorrect to suppose that the enzyme centre, or the active portion constituting the enzyme, necessarily possesses both basic and acidic groups, even though the reaction is presumably a hydrating one and a symmetrical p_H-activity curve is evinced [see also Woolf, 1931]. It seems not unreasonable to suppose that the p_H-activity curve may be influenced by other factors, besides the enzyme proper, which are subject to change on variation of p_H. Such factors may, for example, be the constitution of the substrate molecule, or the colloidal carrier to which the active centre constituting the enzyme is attached.

Influence of unsaturated glycerides on the toxicity of basic triphenylmethane dyes.

As stated earlier the addition of a substance, which is precipitated from a soya bean preparation by CO_2, to the purified enzyme restores the susceptibility of the enzyme to the toxic action of brilliant green.

A preparation of urease was made from jack bean meal[1]. 10 g. of this meal were shaken with 90 cc. distilled water for 30 mins. and centrifuged, and the centrifugate was used as the source of the urease preparation. This proved to be fifteen times more active than a soya bean preparation made in the same way. It was diluted with water twenty times, preparatory to use. This preparation of urease was found to resemble purified urease, obtained from soya bean, in so far as susceptibility to dyestuffs was concerned. Brilliant green and crystal violet had but little toxic action on the enzyme at low concentrations; the addition to it, however, of a little of the soya bean preparation brought about a great enhancement of the toxicities of these dyestuffs. The same phenomenon occurred with a preparation of crystalline urease, obtained from jack bean meal by Sumner's method [1926]. Here the addition of 0·25 cc. of a soya bean preparation of urease to a solution of crystalline urease caused brilliant green at a concentration of 1/50,000 to remove 90 % of its activity. This concentration of brilliant green had no effect on the activity of the solution of crystalline urease itself.

Extracts were made from a variety of other vegetable sources but soya bean seemed to be unique in its possession of a co-toxic factor for the basic triphenylmethane series of dyes.

It was then found that an ether extract of soya bean contained the co-toxic factor, soya bean carefully extracted with ether no longer being susceptible to the toxic action of brilliant green. On suspending a little soya bean oil in a purified urease preparation, the ability of a low concentration of brilliant green to inactivate 90 % of the enzyme was restored. Subsequent examination

[1] Kindly supplied me by Dr J. B. Sumner.

of very many oils showed that the faculty of restoring toxicity to brilliant green belonged apparently to unsaturated glycerides, linseed oil in particular being very efficient. Thus 1/50,000 crystal violet had no toxic action on a urease preparation from jack bean meal; on adding to the mixture of enzyme and dyestuff linseed oil to give a final concentration of 0·05 %, 91 % inhibition of the activity of the enzyme was effected. The linseed oil alone had no toxic action on the enzyme. The linseed oil was used as a 1 % alcoholic solution, 0·5 cc. of this being added to the solution containing enzyme and dyestuff.

Table III shows the effects of olive, linseed and cod-liver oils on the toxicity of brilliant green to jack bean urease.

Table III. *Percentage inhibition of the activity of jack bean urease by brilliant green (1/50,000) in the presence of various oils (0·05 %).*

Initial incubation period, 30 minutes.

Oil	Percentage inhibition
Olive	10
Linseed	66
Cod-liver	28

The co-toxic action of linseed oil *etc.* applies apparently only to the basic triphenylmethane series of dyes. They have little or no effect on the toxicity of neutral red or methylene blue; nor do they enable acidic dyestuffs to become toxic. The mode of action of the unsaturated glycerides in promoting this toxicity is a problem for further investigation. They appear to behave as highly specific mordants between the enzymes and the dyestuff. They do not enhance the toxic effects of dyestuffs on another enzyme, fumarase—so that their specificity extends so far as is known at present to urease on the one hand and to the triphenylmethane series of basic dyes on the other.

It is probable that these results have a considerable bearing on the well-known differential antiseptic actions of the triphenylmethane series of basic dyes.

Effects of admixture of acid and basic dyestuffs.

It was shown in the earlier work on fumarase [Quastel, 1931] that addition of acid dyestuffs to toxic basic dyestuffs (and *vice versa*) led to the disappearance of toxicity. This was due, presumably, to mutual combination or adsorption between the oppositely charged dyes.

This phenomenon may be demonstrated also using urease. Owing to the fact that the dyes of the basic triphenylmethane series are extremely toxic at very low concentrations (1/100,000) to soya bean urease, it is difficult to show the reversing action of acid dyes with this preparation of enzyme. It is comparatively easy, however, to demonstrate the phenomenon with the purified enzyme to which the dyes are less toxic at low concentrations.

Ethyl violet (1/10,000) gave a 76 % inhibition of the activity of a preparation of jack bean urease. On adding to the ethyl violet trypan blue solution

(1/10,000), allowing to stand for 10 mins. and then adding the enzyme, the percentage inhibition was 18 %. The initial incubation period prior to addition of urea was 30 mins. Reversibilities of a similar kind were effected by benzopurpurin, orange G and crystal scarlet solutions at a concentration of 1/10,000.

There seems little doubt from these results that the basic dyes effect their toxicity by combining with an acidic or negatively charged group in the active centre constituting the enzyme. So far as is known at present the combination is irreversible.

Urea protects urease from the toxicity of the dyes. This is easily shown by adding urea at the same time as the dye to the enzyme. The ammonia liberation in such circumstances is considerably greater than when the urea is added after an initial incubation period of the enzyme with the dyestuff alone.

Thus the NH_3 liberation in 1 hour from urea (10 cc. 0·3 %) when this was added to urease + brilliant green (1/50,000) after 1 hour's initial incubation was 0·52 cc. $N/10$, whereas from the same quantity of urea when this was added to the enzyme and dyestuff without any initial incubation period it was 5·82 cc. $N/10$.

It has been found that many substances resemble urea in protecting urease from the toxic dyestuffs.

Protection of urease from the toxic action of brilliant green.

Much of the work on the protection of urease by various substances has been carried out with brilliant green as the toxic dyestuff. What applies to brilliant green, however, applies also qualitatively to the other toxic dyestuffs.

The enzyme was incubated at p_H 7·4 in the presence of brilliant green (usually at a concentration of 1/50,000) and the substance under investigation at 45° for an initial incubation period of 30 mins. or 1 hour. 1 cc. 3 % urea was then added to the mixture, which was finally incubated for 1 hour, after which the ammonia formed was estimated. This value was compared with that produced in the absence of the protective substance. If the inhibition was reduced from 90 % to 50 % by the addition of the substance, the protection was defined as $90 - 50 = 40$ %.

Table IV shows the percentage protections afforded by a number of substances towards soya bean urease against brilliant green.

It will be noted that the amino-acids and basic amines have considerable protective actions, whilst amides, or molecules in which the amino-group has been linked directly with the carbonyl group, are inert. Glycine is very active but hippuric acid is inert; aspartic acid is active but its near non-nitrogenous relatives, succinic, fumaric and malic acids are without action. This may be contrasted with the protective actions exhibited by fumaric and succinic acids towards fumarase against toxic dyestuffs [Quastel, 1931].

In considering, in particular, the protective action of glycine it seems possible that the protection may be due to combination between the amino-

Table IV. *Effects of various substances on the toxicity of brilliant green (1/50,000) towards urease (soya bean preparation).*

Initial incubation period, 30 mins. at 45°. All acids were neutralised with NaOH, the p_H of all solutions prior to addition of urea being 7·4.

Substance		Protection %	Substance		Protection %
Glycine	0·04 *M*	58	Hydroxylamine	0·04 *M*	23
p-Aminobenzoic acid	,,	29	Semicarbazide	,,	43
Aspartic acid	,,	49	Allantoin	,,	60
Glutamic acid	,,	60	Ethylurethane	0·08 *M*	0
Succinic acid	,,	0	Hippuric acid	0·04 *M*	0
Fumaric acid	,,	0	Oxamic acid	,,	0
Malic acid	,,	0	Betaine	,,	0
Ethylenediamine	0·08 *M*	92	Methylurea	,,	1
Hydrazine	0·04 *M*	47	Thio-urea	,,	5

acid and the dyestuff. This possibility may be dismissed, however, on the following evidence. Ethyl violet is toxic to urease (soya bean) at a concentration of 1/100,000. Glycine (0·04 *M*) protects the enzyme from the toxicity of the dye even when the concentration of the latter is increased to 1/10,000. Assuming that the protection is due to combination between the amino-acid and dyestuff, it must be concluded that one part of 0·04 *M* solution of glycine combines with one part of at least a 9/100,000 solution of ethyl violet. Now it may be shown that 1/50,000 ethyl violet solution has a very slight toxicity to fumarase and that 1/10,000 ethyl violet solution is completely toxic, no protection being afforded by the presence of 0·08 *M* glycine. If it is true however that one part of 0·04 *M* glycine combines with 9/100,000 ethyl violet solution, one part of 0·08 *M* glycine will combine with a larger quantity than one part of 1/10,000 ethyl violet solution and hence should completely protect fumarase. The reverse of this is true and hence it cannot be assumed that protection of the enzyme is due to combination between glycine and the dyestuff.

The protective action of glycine is greater, the smaller the quantity of dyestuff present. This is shown in Table V.

Table V. *Protection by glycine against toxic effects of brilliant green on urease (soya bean preparation).*

Initial incubation period of mixture of enzyme, dye and glycine at 45° was 30 mins. Figures show cc. of *N*/10 NH_3 produced in 1 hour. Maximum obtainable = 10 cc.

Glycine concentration	Concentration of brilliant green		
	1/16,660	1/25,000	1/100,000
0	0·28	0·00	0·3
0·02 *M*	1·12	1·62	7·36
0·04 *M*	1·84	2·91	8·50
0·06 *M*	2·20	3·41	8·79

The protective effects of amino-acids and basic amines are shown equally well with a jack bean urease preparation or with a solution of the crystalline enzyme, 0·05 % linseed oil having been added to these preparations. Typical results are shown in Table VI where the figures quoted represent amounts of

ammonia produced after 1 hour's final incubation, given as percentages of the total quantity of ammonia obtainable (*viz.* 10 cc. $N/10$).

Table VI. *Effects of various substances on toxicity of brilliant green (1/50,000) towards urease (jack bean) in presence of 0·05 % linseed oil.*

Initial incubation period, 60 minutes.

Substance	Molar conc.	Percentage production of NH_3	Substance	Molar conc.	Percentage production of NH_3
Glycine	0·013	76	Hippuric acid	0·02	0
Sarcosine	0·011	71	Methylurea	0·027	7
Methylamine	0·03	40	Biuret	0·02	20
Dimethylamine	0·04	61	Thio-urea	0·027	7
Trimethylamine	0·04	0	Guanidine	0·033	6
Creatine	0·027	20	Diethylurea	0·033	0
Betaine	0·035	2	Formamide	0·2	0

REVERSIBILITY OF THE COMBINATION BETWEEN GLYCINE AND UREASE.

It appears that the union between urease and glycine is reversible, the law of mass action being followed. This may be shown as follows.

Let the initial concentration of urease be E and that of glycine be G. Let the concentration of the glycine-enzyme complex at equilibrium be EG.

Then the concentration of free enzyme at equilibrium $= E - EG$, and the concentration of glycine at equilibrium $= G - EG$. At equilibrium we have

$$K' = \frac{EG}{(E-EG)(G-EG)} = \frac{EG}{G(E-EG)}$$

since EG is taken as small compared with G.

Therefore
$$EG = \frac{K'E.G}{1+K'G}$$

where K' is a constant, the reciprocal of the dissociation constant of the glycine-enzyme complex.

Now the amount of urea-enzyme compound must be proportional to EG for that quantity of enzyme which has not combined with glycine has been almost entirely attacked and rendered inert by the dyestuff in the initial incubation period. Hence the amount of enzyme available to attack urea at the end of this period = constant EG = constant $\frac{E.G}{1+K'G}$.

The amount of ammonia produced will be proportional to the amount of enzyme available to attack the urea and hence

$$A = NH_3 \text{ produced} = \text{constant}\,\frac{E.G}{1+K'G}.$$

Keeping the initial quantities of enzyme and dyestuff constant and given an excess of urea, we should find that

$$A = \frac{\text{constant } G}{1+K'G}$$

or
$$A = \frac{\text{constant } G}{K+G}$$

where K = dissociation constant of the urease-glycine complex.

Experimental results are given in Fig. 1, where the curve drawn is

$$A = \frac{0{\cdot}086\,G}{0{\cdot}0153 + G}.$$

A being expressed as molar concentration of ammonia produced and G as the initial molar concentration of glycine present. In Fig. 1 A is plotted against $-\log G$. The experimental results are denoted by crosses and it will be seen

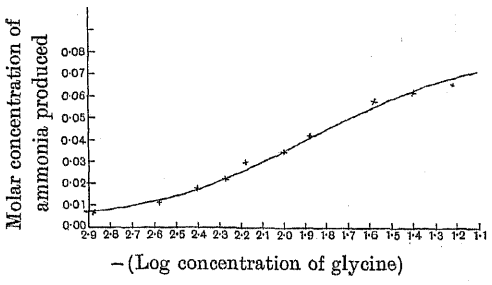


Fig. 1.

that the curve expresses, within the limits of experimental error, the observed variations in the concentrations of ammonia produced with the changes in initial glycine concentrations[1]. The constants of the equation were obtained from two points on the curve. These give 0·0153 as the dissociation constant of the glycine-enzyme complex, but this must be considered as approximate (though of the correct order of magnitude) in view of the possibility that some further destruction of the enzyme occurs by means of the dyestuff during the final incubation period. The probability is that the correct dissociation constant of the glycine-enzyme complex is a little lower than 0·0153.

If the initial concentrations of glycine and dyestuff be kept constant A should vary directly with E. This is found to be true within the limits of experimental error.

Nature of the groupings responsible for combination with urease.

It is evident from a study of Tables IV and VI that the groupings responsible for the attachment of a substrate molecule to urease consist of the primary amino-group and the secondary imino-group.

Molecules containing the primary amino-group which show protection are glycine, aspartic and glutamic acids, arginine and histidine (see Fig. 2), methylamine, ethylenediamine, hydrazine, hydroxylamine, semicarbazide and *p*-aminobenzoic acid.

Molecules containing the imino-group which show protection are sarcosine and dimethylamine.

Molecules possessing the $N(CH_3)_3$ group which have no protective effects are betaine and trimethylamine.

[1] Brilliant green, 1/50,000, was used as the toxic dyestuff.

Molecules containing a —CO—NH_2 or a —CO—NH-linkage which have no protective action are oxamic acid, hippuric acid, ethylurethane, diethylurea and formamide.

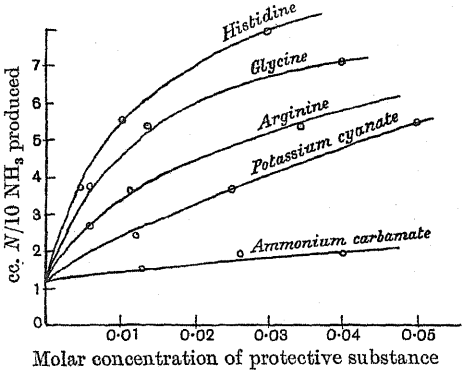


Fig. 2. Effects of amino-acids, potassium cyanate and ammonium carbamate on NH_3 production by urease in presence of brilliant green (1/50,000).

Thio-urea, guanidine and methylurea have very little protective action at the concentrations tested, whereas creatine, biuret and allantoin have decided effects. It is evident that protective effects with substituted ureas depend greatly on the chemical nature of the substituents.

It is necessary at this juncture to mention that in working with crystalline or highly purified preparations of urease sometimes the reverse of protective effects are obtained—particularly with hydrazine and hydroxylamine. These substances may themselves show considerable toxicity towards the enzyme. This effect is in all probability due to the presence of metals (? Cu), for their toxicity disappears entirely in the presence of a trace of potassium cyanide. Potassium cyanide itself shows no protective action towards urease from brilliant green, but in its presence both hydroxylamine and hydrazine show powerful protective effects to the purified enzyme.

It seems clear from the results as a whole that protective action is associated with the presence of basic amino- or imino-groups. These allow the protective molecules to combine reversibly with the acidic or negatively charged groups which form, either wholly or partially, the active centre of urease. With these groups basic dyes appear to combine irreversibly. Presumably urea is combined also with these groups, activation occurring after combination has taken place.

Protective action of potassium cyanate.

It is taken for granted that since an enzyme catalyses a reversible reaction in both directions, the end products, as well as the initial substances, have some affinity for the enzyme. In view of the well known controversy as to whether cyanic acid or carbamic acid is formed from urea by the action of urease it was of interest to determine whether either or both these substances

combined with the enzyme. If combination occurred, clearly some protection would be afforded against a toxic dyestuff.

Experiment showed that ammonium carbamate had little or no protective action, whilst potassium cyanate had a very definite protective effect. Control experiments were carried out to take into account any spontaneous breakdown of cyanate itself into ammonia. The results are shown in Fig. 2 where comparative results with glycine, histidine and arginine are also given. It will be seen that the affinity of cyanate is less than that of glycine but out of all proportion greater than that of ammonium carbamate. The fact that carbamic acid has no protective action is in accord with results already recorded which show that molecules containing the carbamide linkage are usually inert. The definite protective action of cyanate shows that combination with the enzyme does occur with this substance and is evidence in favour of the view that cyanic acid, rather than carbamic acid, is formed from urea by urease. Sodium thiocyanate, it is worthy of note, is entirely without protective action.

Summary.

1. The action of 23 dyestuffs on urease, prepared from soya bean and jack bean, has been investigated. Acidic dyes are entirely inert. Most basic dyes are toxic.

2. The triphenylmethane series of basic dyes is highly toxic to a soya bean preparation of urease, brilliant green being toxic at a concentration of 1/100,000. This toxicity diminishes and may disappear at low concentrations on purifying the urease preparation. The toxicity of other dyestuffs such as Janus green or neutral red increases with increased purification of the enzyme.

3. A substance is present in soya bean which enhances greatly the toxicity of the basic triphenylmethane dyes towards purified urease. This is found to be present in soya bean oil. The property of enhancing the toxicity of brilliant green *etc.* to urease appears to be confined to the unsaturated glycerides. Linseed oil is particularly effective. It seems that the unsaturated glycerides act as highly specific mordants between urease and the basic triphenylmethane dyes.

4. Many substances protect urease from the toxic action of dyes. Such substances are urea, α-amino-acids, sarcosine, ethylenediamine, methylamine, dimethylamine, hydrazine and hydroxylamine.

Trimethylamine, betaine, urethane, methylurea, diethylurea, oxamic acid *etc.* are without effect.

5. Glycine combines reversibly with urease, the dissociation constant of the urease-glycine complex having been determined.

6. The combining group of the protective substances is either a basic amino-group or imino-group. Combination of α-amino-acids, basic amines *etc.* takes place reversibly at acidic or negatively charged groups which constitute either wholly or partially the active centre of urease. Basic dyes appear

to combine with these groups irreversibly. Urea is combined at these groups, activation of the molecule then occurring.

7. Potassium cyanate protects urease from the action of brilliant green; ammonium carbamate has little or no effect. This is evidence for combination between cyanic acid (but not carbamic acid) and the enzyme, and supports the contention that cyanic acid is produced from urea by urease.

I wish to express my indebtedness to the Medical Research Council for a grant in aid of the equipment of this laboratory.

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CCI. THE ACTION OF BONE PHOSPHATASE ON THE ESTERS OF PHOSPHORIC ACID OF THE BLOOD.

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THE presence in bone of an enzyme capable of hydrolysing organic esters of phosphoric acid to yield inorganic phosphate was demonstrated by Robison [1923]. A considerable body of evidence has since been brought forward by Robison and his co-workers in support of the suggestion that the bone enzyme is intimately concerned in the process of ossification in bone formation. It was suggested that the deposition of bone salts resulted from the hydrolysis by the enzyme (phosphatase) of organic compounds of phosphorus to yield free phosphate, any increase of which would, in the presence of inorganic calcium, lead to precipitation of calcium phosphate. The presence, in the fluid bathing the bone, of a suitable substrate for the phosphatase was, of course, a necessary part of this hypothesis, and Kay and Robison [1924] were able to demonstrate a partial hydrolysis in the presence of the enzyme of the "acid-soluble organic phosphorus" of the blood. Their experiments were carried out on neutralised trichloroacetic acid filtrates of whole blood, on which the bone phosphatase was allowed to act at 37° and at its optimum p_H of about 9. Under these conditions only a fraction (usually about 25 %) of the acid-soluble organic phosphorus compounds present in the blood was hydrolysed by the enzyme. Since dilute solutions of primary esters of phosphoric acid are readily and almost completely hydrolysed by phosphatase in a few hours, it was supposed that in blood there were at least two phosphoric esters, one of which was rapidly hydrolysed by bone phosphatase, while the other was very resistant to the action of the enzyme. In contrast to the limited hydrolysis by enzymic action, almost complete hydrolysis of the acid-soluble phosphorus of blood resulted from boiling with dilute mineral acid.

In some experiments in this laboratory on the combined action of phosphatase and lipase on lecithin, an unexpectedly large increase of inorganic phosphorus was observed in control flasks which contained phosphatase, lipase and buffer, but no lecithin. The source of this phosphorus was traced to the lipase preparation, an extract of pig's pancreas, which was found to contain large amounts of acid-soluble phosphorus. It seemed worth while to investi-

gate the effect of phosphatase on these phosphorus compounds contained in the pancreas, and the action of the enzyme on neutralised acid extracts of the pancreas was accordingly studied. At p_H 9, the optimum hydrogen ion concentration for phosphatase, about a quarter of the total acid-soluble phosphorus was liberated. At or near p_H 7, however, a somewhat larger proportion, corresponding to about 50 % of the organic phosphorus present, was hydrolysed under the influence of the enzyme during 24 hours.

With acid extracts of liver, kidney and muscle the p_H effect was not so marked, maximum hydrolysis being effected under the action of the enzyme at a p_H intermediate between 7 and 9. In the case of blood, only relatively small proportions of the acid-soluble phosphorus were hydrolysed at the optimum p_H of the enzyme, as was found by Kay and Robison. The hydrolysis proceeded, however, to a much greater extent, if the reaction of the mixture was maintained near neutrality. In some instances over 90 % of the phosphoric esters contained in the acid-soluble fraction of whole blood were hydrolysed by the bone phosphatase at or near p_H 7. Not much more than this amount of hydrolysis was obtainable even under the action of boiling $N/2$ sulphuric acid.

Experimental.

In the enzymic hydrolysis of lecithin the method used [King, 1931] for estimating the "lecithinase" activity of a tissue extract consisted in measuring the increase of inorganic phosphate, during 48 hours at 37°, in mixtures of buffered lecithin and tissue extract. Since it has been demonstrated that lipase acts on lecithin as well as on neutral fat to liberate fatty acids [Contardi and Latzer, 1927; Paal, 1929], it seemed possible that the combined action of lipase and lecithinase might lead to a greater increase of free phosphate than the action of lecithinase alone. The first lipase preparations used were crude extracts of pig's pancreas made according to Cole [1928] by treating finely minced pancreas with aqueous alcohol. This extract shows a high lipolytic activity for olive oil and other neutral fats, particularly in the presence of bile salts. When 1 cc. of Cole's lipase solution was added together with 2 cc. of a preparation of lecithinase (rabbit's intestine) to 10 cc. of a buffered 0·25 % solution of lecithin there was an increase in inorganic phosphate (F.P.) of 0·127 mg. P in 48 hours, while 0·111 mg. F.P. were liberated by the action of the lecithinase alone. If a preparation of bone phosphatase was substituted for the rabbit's intestinal extract there was practically no increase of F.P. due to the phosphatase alone, whereas the addition of 1 cc. of lipase solution led to an increase of 0·139 mg. in the case of a 10 % rabbit's bone extract and of 0·118 mg. in the case of an extract of rachitic chicken bones. The increase of total acid-soluble phosphorus was, however, very small in all cases, there being a large amount present in the initial trichloroacetic acid filtrates of mixtures containing lipase. The increase of F.P. was mainly due, therefore, to hydrolysis of this organic phosphorus (O.P.) of the lipase solution rather than to

hydrolysis of the lecithin. Controls consisting of the same proportion of rabbit's or chicken's bone phosphatase, lipase, and buffer (with no lecithin solution) showed increases of 0·114 mg. and of 0·099 mg. F.P. during the same time at 37°, which is almost as much as that obtained when lecithin was present.

This increase of free phosphate at the expense of the acid-soluble phosphorus of pig's pancreas appeared to demonstrate the presence of phosphoric esters capable of acting as a substrate for phosphatase. Primary esters of phosphoric acid are optimally hydrolysed at p_H 9 [Robison, 1923], secondary esters at p_H 7·4 to 7·6 [Asakawa, 1929] and pyrophosphate at p_H 7·2 to 7·8 [Kay, 1928]. A study of the effect of hydrogen ion concentration on the hydrolysis of the phosphoric esters of pancreas by bone phosphatase indicated that maximum hydrolysis occurred between p_H 7·0 and 7·5. Cole's lipase extract of pig's pancreas (90 cc.) was treated with 10 cc. of 25 % trichloroacetic acid, and the precipitate filtered off. 40 % sodium hydroxide was added drop by drop to the filtrate until pink to phenolphthalein and the solution filtered from a further precipitate. For the hydrolysis, test solutions of 10 cc. of the neutralised trichloroacetic acid filtrate, 10 cc. of buffer and 5 cc. of a dilute phosphatase solution were kept at 37° and 6 cc. portions withdrawn at intervals for the determination of free phosphate by the Martland and Robison colorimetric procedure [1926]. The results are illustrated in Table I.

Table I. *Phosphatase hydrolysis of the acid-soluble phosphoric esters of pig's pancreas.*

	F.P. liberated		
	4 hours	8 hours	24 hours
p_H	mg.	mg.	mg.
5·7	0·011	0·015	0·046
6·6	0·018	0·036	0·111
7·0	0·059	0·095	0·172
7·5	0·080	0·125	**0·196**
8·7	0·054	0·083	0·137
9·2	0·044	0·068	0·115

O.P. = 0·420 at zero time; maximum hydrolysis (24 hours) = 47 %.

Fresh dog's liver (64 g.) was removed from an anaesthetised (sodium amytal) animal and ground immediately in 250 cc. of ice-cold 5 % trichloroacetic acid. The filtrate was neutralised with 6 cc. of 40 % sodium hydroxide and a further precipitate removed. Test solutions consisted of 5 cc. of the extract, 5 cc. veronal buffer [Michaelis, 1930], 2·5 cc. of 10 % rat's bone extract, acid to give the desired p_H and water to 25 cc. Each 5 cc. of this mixture represented 1 cc. of liver extract and 0·5 cc. of bone phosphatase solution. Portions of 5 cc. were removed at intervals for estimations of free phosphate. For the acid hydrolysis at 100° a mixture of equal parts of liver extract and *N* sulphuric acid was kept under a reflux condenser in a boiling water-bath. The kidneys and portions of muscle (sartorius) were removed from the same

dog and treated as in the case of the liver. The results of these experiments are illustrated in Table II.

Table II.

Hydrolysis of the phosphoric esters of dog's liver.

Initial O.P. (in 1 cc. of trichloroacetic extract) = 0·152 mg.

	8 hours	24 hours	48 hours	72 hours
	Percentage hydrolysis			
p_H				
7·0	12	14	18	30
7·1	11	18	21	29
7·5	14	22	25	36
8·3	18	28	34	49
9·1	9	18	29	45
9·7	7	10	16	24
$N/2$ H_2SO_4	27	52	76	84

Hydrolysis of the phosphoric esters of dog's kidney.

Initial O.P. (in 1 cc. of trichloroacetic extract) = 0·067 mg.

	8 hours	24 hours	48 hours	72 hours
	Percentage hydrolysis			
p_H				
6·4	16	45	33	36
6·9	21	46	45	48
7·3	28	58	51	54
8·0	31	60	52	55
8·9	25	54	48	48
9·6	—	4	13	27
$N/2$ H_2SO_4	18	52	82	97

Hydrolysis of the phosphoric esters of dog's muscle.

Initial O.P. (in 1 cc. of trichloroacetic extract) = 0·081 mg.

	8 hours	24 hours	48 hours
	Percentage hydrolysis		
p_H			
6·2	9	—	33
7·0	31	57	81
7·4	47	70	88
8·2	59	80	94
9·0	35	61	65
9·6	5	12	28
$N/2$ H_2SO_4	40	69	84

Mixed whole blood from the carotid artery and femoral vein of this dog was laked with 3 volumes of water and precipitated immediately with 1 volume of 25 % trichloroacetic acid. The mixture was centrifuged, neutralised with strong sodium hydroxide and filtered. The test solutions for enzyme hydrolysis consisted of 7·5 cc. blood-filtrate, 2·5 cc. bone extract, buffer and water to 25 cc. Aliquot portions (5 cc. equivalent to 1·5 cc. blood-filtrate and 0·5 cc. bone extract) were withdrawn at intervals for free phosphate determinations. The results for this dog's blood and for a rabbit's blood are contained in Table III.

Table III. *Hydrolysis of phosphoric esters of blood.*

Dog's blood; initial O.P. (in 1·5 cc. trichloroacetic acid extract) = 0·066 mg.

	2 hours	12 hours	21 hours	36 hours	60 hours
	Percentage hydrolysis				
p_H					
3·2	—	2	3	6	8
4·2	—	2	3	5	17
5·7	3	5	23	44	55
6·1	5	20	33	62	77
7·0	**5**	**30**	**44**	**68**	**84**
7·5	6	21	33	56	61
8·1	5	11	27	35	41
8·8	3	9	12	21	32
9·2	—	12	21	21	30
$N/2$ H_2SO_4	11	49	55	85	—

Rabbit's blood; initial O.P. (in 1·5 cc. trichloroacetic acid extract) = 0·074 mg.

3·2	3	3	4	7	—
4·7	—	3	4	7	—
5·5	4	9	11	15	16
6·3	4	31	34	55	75
6·9	**3**	**49**	**50**	**66**	**84**
7·4	19	47	49	62	74
7·9	—	35	45	53	57
8·5	23	34	35	42	43
9·1	16	28	32	32	28
9·8	3	12	16	—	16
$N/2$ H_2SO_4	—	39	51	80	92

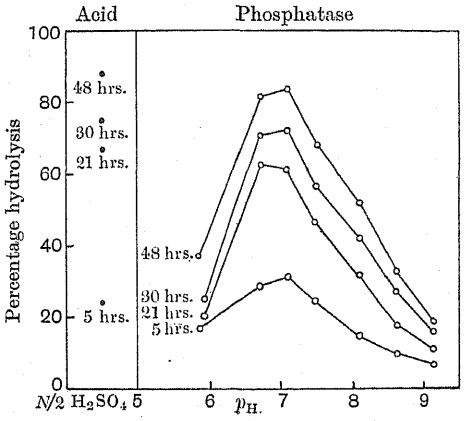


Fig. 1. Hydrolysis by acid and by phosphatase of the "acid-soluble phosphorus" of dog's blood.

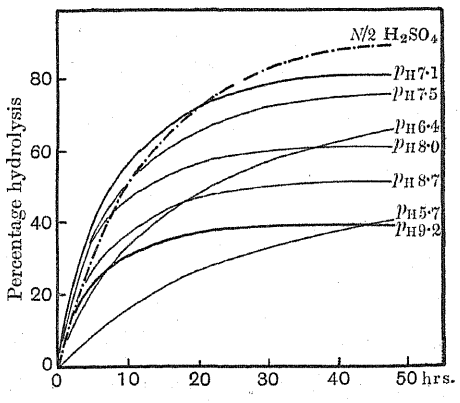


Fig. 2. Hydrolysis by acid and by phosphatase of the "acid-soluble phosphorus" of rabbit's blood.

The hydrolysis of the acid-soluble phosphorus compounds of the blood of another dog is illustrated in Fig. 1 and of that of a rabbit in Fig. 2.

These results with animal blood appeared to indicate that if the hydrolysis were continued long enough, the greater part of the acid-soluble phosphoric esters could be hydrolysed by the bone enzyme at a reaction in the neighbourhood of neutrality. In order to determine if human blood behaved in like manner, samples were taken from the arm veins of five normal adult males. 10 cc. samples were laked with 30 cc. of water and the proteins precipitated

by the immediate addition of 10 cc. of 25 % trichloroacetic acid. After being shaken thoroughly the samples were centrifuged, the supernatant solutions poured off, neutralised by the careful addition of powdered sodium hydroxide, and filtered. 15 cc. portions of the filtrates were added to 25 cc. volumetric flasks, together with 5 cc. of veronal buffer and 2·5 cc. of 5 % dialysed rat's bone extract. The reaction of each solution was adjusted to p_H 7, and water added to 25 cc. Two drops of a mixture of chloroform and toluene were added and the flasks placed in a water thermostat at 37·5°. At stated intervals 5 cc. portions (equivalent to 3 cc. of trichloroacetic acid filtrate of blood and 0·5 cc. enzyme) were withdrawn and the inorganic phosphate was determined. The results (Table IV) show that the acid-soluble phosphorus of human blood, although attacked relatively slowly by the bone enzyme under these conditions, is, nevertheless, almost completely hydrolysed in a little less than 2 days.

Table IV. *Acid-soluble phosphorus of human whole blood.*

10 cc. blood + 30 cc. water + 10 cc. 25 % trichloroacetic acid. Hydrolysis of 3 cc. neutralised filtrate (equivalent to 0·6 cc. blood) by 0·5 cc. rat's bone extract at p_H 7 and 37·5°.

	O.P. mg.	F.P. 0 hours mg.	F.P. 6 hours mg.	F.P. 12 hours mg.	F.P. 24 hours mg.	F.P. 40 hours mg.	Increase after hydrolysis mg.	Percentage hydrolysis
H. M. D.	0·122	0·034	0·096	0·120	0·135	0·149	0·115	94
D. A. I.	0·138	0·035	0·101	0·128	0·149	0·155	0·120	87
J. E. W.	0·120	0·034	0·094	0·119	0·136	0·146	0·112	93
W. R. F.	0·128	0·032	0·067	0·120	0·140	0·150	0·118	92
E. J. K.	0·132	0·032	0·102	0·120	0·136	0·154	0·122	92

Since part of this liberated phosphate might be due to spontaneous decomposition of the phosphoric esters, or possibly derived from organic phosphorus contained in the enzyme preparation, an experiment was conducted in which controls consisting of buffer and trichloroacetic acid filtrate of blood, and of enzyme and water were incubated under the same conditions as mixtures of the blood-filtrate, buffer and enzyme. The results contained in Table V demonstrate that with dog's and rabbit's bloods there was no appreciable increase of inorganic phosphate in either of the controls, and that at least

Table V. *Hydrolysis of acid-soluble phosphorus of blood at* p_H *7.*

Hours	F.P. substrate + enzyme + buffer mg.	F.P. substrate + buffer mg.	F.P. enyzme + water mg.	P hydrolysed mg.	Percentage hydrolysis
Dog's blood: 0·152 mg. O.P. in 3 cc. of trichloroacetic acid filtrate.					
0	0·035	0·025	0·009	0·001	—
17	0·102	0·031	0·013	0·058	38
47	0·161	0·029	0·010	0·122	80
Rabbit's blood: 0·165 mg. O.P. in 3 cc. of trichloroacetic acid filtrate.					
1	0·034	0·016	0·011	0·007	4
24	0·143	0·016	0·013	0·114	69
48	0·161	0·017	0·011	0·133	81
72	0·167	0·018	0·011	0·138	84

four-fifths of the organic phosphorus contained in the acid extracts of these bloods was hydrolysed within 2 days by the enzyme.

It seemed worth while to test the susceptibility of the residual phosphoric esters, unattacked by the enzyme at p_H 9, to bone phosphatase at a lower p_H. 15 cc. samples of human blood-filtrate were adjusted to p_H 9 and maintained at 37·5° in the presence of 2 cc. of bone extract for 24 hours, when equilibrium had apparently been reached with the hydrolysis of some 35 % of the organic phosphorus. At this point 2 cc. more of enzyme were added to each of the flasks, since that originally present had probably been largely destroyed during the 24 hours at an alkaline reaction. Two flasks were adjusted to p_H 7 and two maintained at p_H 9 and further incubated at 37·5°, aliquot portions being withdrawn at intervals for inorganic phosphate and total phosphate determinations. The differences (organic phosphate) indicated that there was only 5 % of organic phosphate left in the mixtures at p_H 7 after 36 hours, while at p_H 9 there was still nearly 60 % unattacked. The results are contained in Table VI.

Table VI. *Hydrolysis of residual phosphoric esters after hydrolysis at* p_H *9.*

20 cc. human blood + 60 cc. water + 20 cc. 25 % trichloroacetic acid: 0·129 mg. O.P. in 3 cc. filtrate. Preliminary incubation with enzyme for 24 hours at p_H 9.

	O.P. at p_H 9		O.P. at p_H 7	
Hours	mg.	Unhydrolysed %	mg.	Unhydrolysed %
0	0·084	65	0·083	64
10	0·083	64	0·054	42
24	0·079	61	0·024	19
36	0·075	58	0·007	5
50	0·073	57	0·006	5
		O.P. hydrolysed 43		O.P. hydrolysed 95

Summary.

The esters of phosphoric acid contained in trichloroacetic acid extracts of blood consist of at least two fractions. One is attacked by bone phosphatase at p_H 9, while the other is hydrolysed preferentially at a p_H near neutrality. Both fractions are almost completely hydrolysed at or near neutrality during 2 days at 37·5°.

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CCII. THE ISOLATION OF GLUTAMINE FROM AN ENZYMIC DIGEST OF GLIADIN.

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The recent isolation of asparagine from an enzymic digest of edestin [Damodaran, 1932] provided the first direct evidence for the existence of a dicarboxylic acid amide in the protein molecule. As was pointed out in that paper it is, perhaps, from the point of view of demonstrating the validity of the amide hypothesis more important to isolate glutamine, because the majority of proteins on acid hydrolysis yield much larger amounts of glutamic acid than aspartic acid. But glutamine was known to be unstable in aqueous solution [Chibnall and Westall, 1932] so that at the end of the period required for the digestion of a protein to the amino-acid stage only a portion of the glutamine set free would still be present as such in the digest. Furthermore, the work of Schulze on the isolation of glutamine from plant extracts suggested that the separation of this amide from the digest would be difficult, and it was for these two reasons that the isolation of asparagine, which is stable in aqueous solution, and which crystallises readily in the presence of other amino-acids, was first attempted. With the experience thus gained a successful attempt has now been made to isolate glutamine from an enzymic digest of gliadin. This protein was chosen because of the high proportion of glutamic acid which it gives on acid hydrolysis; in terms of total protein-N it contains 25·7 % of amide-N, 22·9 % of glutamic acid-N, 1·17 % of hydroxyglutamic acid-N, and only 0·5 % of aspartic acid-N. In other words, if the validity of the peptide and amide hypotheses be assumed the gliadin molecule should yield 42·2 % of glutamine.

At the outset an unforeseen difficulty arose, in that this prolamine was not as susceptible to the action of proteolytic enzymes as, for example, the edestin used in the asparagine research. While with this, and many other of the better known proteins, the successive action of pepsin, trypsin and either intestinal erepsin or yeast dipeptidase could accomplish the liberation of 90 % of the amino-N obtained on complete hydrolysis [Damodaran, 1932; Frankel, 1916], with gliadin it was found that the same enzymes split little more than 70 % of the total peptide bonds. The phenomenon, which invites further

investigation, is undoubtedly connected with peculiarities of molecular structure which must be responsible for the distinctive behaviour of the prolamines as a class and no appreciable improvement in the degree of hydrolysis was attained by variations in the conditions of digestion such as changes in p_H or previous denaturation of the protein.

Since the deficiency in action fell specially on pepsin, which produced a liberation of only about 10–12 % of peptide bonds compared with the 20 % obtained with edestin, it was thought that it might with advantage be replaced or reinforced by a protease of vegetable origin. Papain was accordingly tried, both instead of and in succession to pepsin. No use could, however, be made of the definitely larger hydrolysis thus obtained, as it was found to be accompanied by a simultaneous liberation of large quantities of ammonia, showing that the enzyme preparation was contaminated with an amidase which hydrolysed the amide group of the glutamine. It was necessary, therefore, to use the same three enzymes which had been employed in the digestion of edestin regardless of the much smaller hydrolysis effected.

In carrying out the digestion the procedure adopted followed closely that of the previous investigation, with such small modifications as had been suggested by previous experience or were called for on account of the difference in properties between glutamine and asparagine. In the former research, for instance, the optimum period for the action of each enzyme could be readily ascertained from determinations of amino-N and free ammonia-N. In the present case the instability of glutamine in solution suggested the advisability of determining also the glutamine amide-N. As will be seen from the tables given later this precaution was fully justified, because in the later stages of the digestion, when splitting of the peptide bonds was approaching a maximum, the rate of decomposition of the glutamine already present in the digest exceeded that at which glutamine was being set free from amino-complexes.

The determination of glutamine by this indirect method was also necessary for another purpose, *viz.* the calculation of the percentage hydrolysis at different stages. The usual procedure is to compare the Van Slyke amino-N present in the digest at any stage with the total amino-N obtained on complete hydrolysis. But as was first clearly pointed out by Chibnall and Westall, glutamine behaves abnormally with nitrous acid, both amino- and amide-groups being decomposed, giving nearly twice the theoretical amino-N value. Therefore, in a digest rich in glutamine, such as would be obtained from gliadin, the Van Slyke amino-N values cannot be accepted as a true measure of the extent of the peptide splitting. An approximate value for this can, however, be obtained by deducting an amount equal to the glutamine amide-N present, as determined by the method of Chibnall and Westall [1932]. It is to be remembered that these workers intended this method, which is based on the rapid liberation of ammonia from glutamine in solution when heated at 100° for 3 hours at either p_H 4 or p_H 8, to be used primarily for the determination of the glutamine content of plant extracts, from which glutamine

peptides have not yet been isolated. They showed, however, that synthetic glutamine peptides of the type

$$R.CH(NH_2).CO.NH.CH(CH_2.CH_2.CO.NH_2).CO_2H$$

were stable. Glutamine peptides of the type

$$R.CH(CO_2H).NH.CO.CH(NH_2).CH_2.CH_2.CONH_2,$$

however, have not yet been synthesised, so that their stability is not known, but as the glutamine amino-group, which is in the γ-position to the amide group, is free, it is possible that they may yield ammonia under the prescribed conditions, and also give abnormal values for Van Slyke amino-N. Now it is probable that peptides of both these types are present in an enzymic digest of gliadin, so that the glutamic amide-N and corrected amino-N values quoted in this paper can have only approximate significance.

The digest of 150 g. of gliadin, from which glutamine was finally isolated, contained initially 27·07 g. of N. At the end of the digestion period the corrected amino-N value was only 69·7 % of that given on acid hydrolysis, so that more than 30 % of the peptide bonds were still intact. Of the amide-N given on complete hydrolysis 31·1 % was present as free ammonia-N and 42·9 % as glutamine amide-N, so that about 25 % was still present in stable amides or peptide amides. Assuming that the glutamine amide-N had its origin in glutamine the amount of the latter present in the digest was 28·1 g.

After treatment with phosphotungstic acid and removal of reagents the clarified solution contained only 6·7 g. of N. Allowing for the basic-N content of the protein (10 % of the total N) and the free ammonia-N (7·5 % of the total N) it is clear that the major part of the residual peptide bonds must have been present in compounds predominantly basic in character, which have been precipitated by this reagent. The calculated amount of glutamine in the clarified solution mentioned above was 10·1 g. As the material recovered from the phosphotungstic acid precipitate contained only a relatively small amount of glutamine amide-N, it appears that the major part of the lost glutamine must have been decomposed during the precipitation operations. The clarified solution gave on treatment 65·8 g. of crystalline material, and after intensive fractional crystallisation 3·4 g. of pure glutamine were finally isolated. This yield, though disappointing as a method of preparing glutamine, must be considered satisfactory when it is remembered that only about one-third of the protein taken was present in the solution after removal of the more complex constituents and that by the end of the digestion nearly one-third of the amide-N was converted into free ammonia. The 3·4 g. of material isolated compares not unfavourably with the 10 g. of glutamine indicated in the final solution by the indirect method, considering the difficulties of crystallising glutamine from a mixture of amino-acids of varying degrees of solubility.

With the existing technique it would be impossible either to prepare an enzymic digest of a protein in which all the asparagine and glutamine originally

present in the protein was liberated intact, or to obtain anything approaching a quantitative isolation of these two amides from an amino-acid mixture, so that the question whether the amide groups of dicarboxylic acids constitute the sole source of the ammonia obtained from proteins must still remain open. But attempts to verify, by actual isolation, the existence of alternative sources of ammonia such as the uramino-acids suggested by Lippich and the ureide groups of Schutzenberger have already been made without success. The negative results of these investigations seem indeed to have been one of the reasons for the general acceptance of the amide hypothesis.

Although the existence of amide groups has been tacitly assumed for a long time, little recognition has ever been given to the practical consequences of such an assumption, mainly it seems because of the lack of any direct evidence. Thus in numerous investigations on the enzymic digestion of proteins the amount of ammonia formed has been used, on the one hand, as a measure of the deamidising activity of the tissue preparations or enzymes used [Luck, 1924, 1; Hunter and Smith, 1925], and, on the other hand, as a basis of conclusions on protein structure [Luck, 1924, 2; Clementi, 1931], without taking cognizance of the fact that the ammonia might have its origin not in enzymic activity but in the secondary decomposition of any glutamine present, either during digestion or even in the course of the actual determination of ammonia. It is interesting to note that the data in the literature on the deamidising activity of different tissues is extremely conflicting, and also that Luck and Clementi, using the same kind of evidence, came to exactly opposite conclusions as to the source of ammonia in proteins. The possibility is also worth considering that the extreme discrepancies attaching to the determinations of blood-ammonia may be connected with the presence in blood of glutamine, which may be absorbed into the blood-stream from the intestinal tract along with other amino-acids.

It will also be apparent that in determining the degree of hydrolysis in protein digests the values obtained from Van Slyke amino-N determinations are bound to disagree with those based on other methods, such as titrations or conductivity measurements, and that in investigations of the kinetics of proteolysis or of the equivalence of amino-groups and carboxyl-groups the conclusions will be of little value unless account can be taken of the discrepancies introduced by the presence of glutamine or labile glutamine peptides.

Experimental.

The gliadin was prepared by extraction of wheat gluten with 73 % alcohol in the usual way and pouring the alcoholic solution after concentration *in vacuo* into acetone, when the protein was precipitated as a fine powder without undergoing any denaturation; moisture, 10 %; N (moisture-free), 17·3 %. The enzyme preparations were similar to those used in the previous work [Damodaran, 1932]. The papain employed in some of the preliminary experiments was obtained from Merck.

Ammonia- and amino-N were determined by the methods employed by Damodaran [1932], while glutamine amide-N was determined after heating at p_H 4 for 3 hours by the method of Chibnall and Westall [1932]. As glutamine was shown by the latter workers to give 92 % of the total N as amino-N in the Van Slyke apparatus, the necessary correction has been made in computing the amino-N value of certain of the digests.

Preliminary digestion experiments.

Degree of hydrolysis attainable. The preliminary experiments were made with 5 g. samples of gliadin in a similar way to those previously carried out with edestin [Damodaran, 1932]. A slightly different procedure was, however, adopted to bring the protein into solution in the first instance. The 5 g. of gliadin were suspended in about 200 cc. $N/20$ HCl, or in later experiments sulphuric acid, and 0·25 g. pepsin dissolved in 25 cc. $N/20$ acid added. The mixture was incubated at 37° and when the solution had attained homogeneity (usually after about 18 hours) the volume was made up to 250 cc. at 37°.

Table I summarises the results of a typical experiment.

Table I. *Showing the digestion of gliadin by pepsin, trypsin and yeast-dipeptidase.*

Enzyme	Duration of digestion (days)	Amino-N (% total amino-N)	Ammonia-N (% total amide-N)	Glutamine amide-N (% total amide-N)
Pepsin-HCl	2	10·3	7·8	—
followed by Trypsin	3	42·6	14·5	22·5
followed by Yeast-dipeptidase	3	63·8	22·2	33·3
„	4	71·7	26·7	31·1

It will be seen that on prolonging the digestion in the last stage from 3 to 4 days, though there is an increase in the amino-N liberated, the glutamine amide-N has fallen from 33·3 to 31·1 %.

Effect of using sulphuric acid and baryta in place of hydrochloric acid and sodium hydroxide. The peptic digest with sulphuric acid was obtained in the same way as with hydrochloric acid. Since the little evidence available in the literature concerning the influence of barium ions on tryptic action is of a conflicting nature it was thought advisable to add baryta only to neutrality, remove the barium sulphate and then bring the solution to p_H 8·5 by the addition of sodium hydroxide, thus avoiding the presence of barium in solution. The results obtained showed little significant difference from those already recorded with hydrochloric acid and in all subsequent digests sulphuric acid was therefore used for securing the necessary acidity for peptic action and most of the acid removed by means of baryta before the addition of trypsin. Determinations on the loss of material during precipitation with baryta showed that after two washings in the cold the barium sulphate contained less than 6 % of the total N in the solution.

Digestion with papain. As papain used directly was found to act but slowly on gliadin, the latter was first brought into solution through the action of pepsin in the usual way, the p_H adjusted to 5 by the addition of sodium hydroxide and a solution of Merck's papain then added. When the peptide splitting with papain had reached a maximum the digestion was continued with trypsin in the usual way.

The results (Table II) would indicate that papain either contains an amidase or has itself a deamidising action and is therefore unsuitable for the purposes of the present research.

Table II. *Showing the digestion of gliadin with pepsin, papain and trypsin.*

Enzyme	Duration (days)	Amino-N (% total amino-N)	Ammonia-N (% of total amide-N)	Glutamine amide-N (% of total amide-N)
Pepsin, followed by papain followed by Trypsin	2½ 3	16·3 44·2	19·7 31·9	Nil Nil

Preparation of a large-scale digest.

150 g. of gliadin and 7·5 g. of pepsin were dissolved in 7·5 litres of 0·05 *N* sulphuric acid at 37°. After 1 day's digestion a further 5 g. of pepsin, dissolved in 25 cc. of the acid, was added. Analysis of an aliquot showed that the digest contained 25·05 g. of N, and after complete hydrolysis 14·97 g. of amino-N and 6·38 g. of amide-N. Additional aliquots were removed from time to time and analysed for free ammonia-N, amino-N and glutamine amide-N, as shown in Table III.

Table III. *Showing extent of hydrolysis in the large-scale digest.*

Enzyme	Duration of digestion (hours)	Amino-N (% total amino-N) Directly determined	Amino-N (% total amino-N) Corrected for glutamine present	Ammonia-N (% amide-N)	Glutamine amide-N (% amide-N)
Pepsin	17	9·5	9·5	4·1	Nil
,,	65	10·1	10·1	10·7	Nil
Trypsin	63	54·5	50·1	16·3	20·8
,,	87	60·2	52·0	18·8	24·1
,,	101	55·2	51·1	21·7	19·9
Yeast-dipeptidase	43	73·6	60·4	27·1	44·0
,,	91	82·5	69·7	31·1	42·9

After 65 days' peptic digestion baryta in the form of a thin cream was added to p_H 7, the precipitate of barium sulphate centrifuged off and washed twice with water. The clear liquid and washings were brought to p_H 8·5 by the addition of *N* NaOH and 10 g. of trypsin in aqueous solution together with 30 cc. of tricresol added. The reaction of the solution was checked daily, and *N* NaOH added to maintain the p_H at 8·5. At the end of 2 days a further 5·0 g. of trypsin was added. Tryptic digestion was allowed to proceed 5 days

in all, when the solution (8600 cc.) was brought back to p_H 7·5 by the addition of sulphuric acid. 500 cc. of a preparation of yeast-dipeptidase were then added, followed by a similar quantity the following day. After 71 hours it was found that the glutamine amide-N had reached a maximum of about 43 % of the total amide-N. The 9600 cc. of digest therefore contained at this stage roughly 28·1 g. of glutamine.

Fractionation of the large-scale digest.

The digest was rapidly concentrated *in vacuo* at 40° to 2200 cc. Two aliquots of 100 cc. were then removed to find out the approximate amount of phosphotungstic acid required for complete precipitation. These trials were deemed necessary because a previous large-scale digest had shown that at this stage nearly two-thirds of the nitrogen in solution was precipitated by this reagent, and we were desirous of finding out if this unexpectedly large proportion was due in part to nitrogen occluded or adsorbed on the precipitate. The two precipitates formed in the present case, after thorough washing with the usual phosphotungstic acid wash-solution, were found to have carried down 64 % of the total N, whereas in the previous experiment, in which the washing was not so thorough, the amount was 67 %. There seemed to be no doubt, therefore, that the digests still contained a large proportion of peptides *etc.* which were predominantly basic in character.

In carrying out this operation on the main fraction of the digest it was necessary to bear in mind the great instability of glutamine at the strongly acid and alkaline reactions involved in the precipitation with phosphotungstic acid and the subsequent removal of excess of this reagent with baryta. The 2 litres of solution were therefore divided into four 500 cc. portions, each portion being submitted to all the operations involved as quickly as possible, brought to neutrality and allowed to stand in the refrigerator before the next 500 cc. portion was dealt with. As the precipitates had perforce to be allowed to stand some time before being washed, with possible decomposition of glutamine, the washings from the different precipitates were not combined with the main solution until after analysis. The exact procedure was as follows. 500 cc. of the solution were cooled in ice and 25 % sulphuric acid, also previously cooled in ice, added to a concentration of 4 % by volume. The solution was allowed to regain room temperature and a concentrated solution of phosphotungstic acid added until precipitation was complete. The mixture was centrifuged, the clear liquid was made strongly alkaline with a thin cream of baryta to precipitate excess phosphotungstic acid and again centrifuged. The solution was brought to p_H 7 with sulphuric acid and allowed to stand in the refrigerator while the same operations were carried out in succession on the remaining three portions of the digest. The neutral solutions were then combined and the precipitated barium sulphate was centrifuged off.

The precipitate of barium phosphotungstate was now washed with saturated baryta, the washings made neutral with sulphuric acid, the barium

Table IV. *Showing the results of complete hydrolysis of the large-scale digest at different stages.*

Digest	Total N (g.)	Amino-N		Amide-N	
		Weight (g.)	% total N	Weight (g.)	% total N
Peptic	25·05	14·97	59·8	6·38	26·7
Tryptic	26·55	15·17	57·2	6·23	23·4
Yeast	27·07	17·54	64·8	6·23	23·0

sulphate centrifuged off and washed twice with cold water. This solution was combined with similar washings of the main barium sulphate precipitate.

The main solution and the collected washings were then separately concentrated *in vacuo* until tyrosine and other sparingly soluble material began to crystallise out. The two solutions were allowed to stand overnight at 0°, filtered, and made up to 250 cc. and 100 cc. respectively. The analyses of these two solutions are given in Table V. It will be seen that they contained but little free ammonia, and about the same proportion of glutamine amide-N. The total glutamine amide-N was 0·967 g., equivalent to 10·07 g. of glutamine.

Table V. *Analysis of the main solution and the washings after treatment with phosphotungstic acid and crystallisation of sparingly soluble material.*

	Main solution (250 cc.)		Washings (100 cc.)	
	Weight (g.)	% total N	Weight (g.)	% total N
Total N	4·999	—	1·729	—
Ammonia-N	0·047	0·94	0·036	2·1
Glutamine amide-N	0·747	14·95	0·220	12·7
Amino-N	3·866	77·34	Not determined	

Isolation of glutamine.

The solution and washings were combined, rapidly evaporated *in vacuo* at 40°, and the material separating out collected in four successive fractions. After the removal of the fourth crop of crystals the solution had attained the consistency of a thin syrup and no separation of material took place on standing overnight in the refrigerator. As it was not advisable in the present case to allow the solution to stand for the long periods that had been permissible in the previous work on asparagine, alcohol was made use of at this stage to help crystallisation. The solution was warmed on a water-bath to about 70° and 95 % alcohol added till a slight turbidity was produced (about 2 vols. of alcohol were required). The solution was clarified by further warming, and on standing at 0° overnight separation of crystalline material took place. After filtration the solution was again concentrated *in vacuo* at 40° and the treatment with alcohol repeated. Three fairly large fractions thus separated were in the form of white crystalline powders and were easily obtained dry and free from syrupy material by washing with absolute alcohol and leaving in the vacuum desiccator. The eighth fraction was a buttery solid capable of being dried by repeated washing with alcohol, but hygroscopic. The ninth fraction was obtained only on the addition of a large excess of alcohol and

could not be completely dehydrated even with acetone. A last fraction was obtained by evaporating the alcoholic mother-liquors and repeatedly dehydrating the syrup thus obtained with acetone.

The analyses of the various fractions are given in Table VI.

Table VI. *Analysis of various crystal fractions.*

Fraction	Weight (g.)	Total N %	Glutamine amide-N (% total N)
I	1·2	10·6	3·0
II	7·0	10·4	3·7
III	10·4	12·2	13·2
IV	10·0	11·4	14·8
V	9·5	3·3	27·9
VI	4·4	12·4	28·9
VII	9·0	12·0	14·9
VIII	9·2	12·7	13·4
IX	3·0	11·7	9·3
X	2·1	9·1	5·9

It will be recalled that from the corresponding digest of edestin the asparagine, which is much less soluble in water than the majority of monoamino-acids, and which crystallises readily in their presence, crystallised out early in the fractionation in one well-defined fraction. As was to be expected from the greater solubility of glutamine in water, this amide was, in the present case, fairly evenly distributed among a number of the fractions. A much more laborious fractionation was accordingly found to be necessary before glutamine could be isolated.

Fractions I and II could be neglected as they contained but little amide-N; while fraction V consisted almost entirely of inorganic material. Both on account of their syrupy nature as well as the comparatively small amount of material present in them, fractions IX and X were also excluded. Fractions III and IV, being similar to each other, were combined, as also were fractions VII and VIII. The two combined fractions thus obtained as well as fraction VI were then submitted to a series of fractional crystallisations. The characteristic needle-like appearance of glutamine crystals under the microscope, together with the analytical data for total N and amide-N obtained for each separate fraction aided the separation of the glutamine from the other amino-acids. The richest fractions obtained from the three sources were then combined and recrystallised from water. 3·6 g. of nearly pure glutamine were thus obtained (N = 18·8 %). One further recrystallisation from water gave 3·4 g. of pure glutamine. For analysis it was dried *in vacuo* over phosphorus pentoxide. (Found: C, 41·3; H, 6·9; N, 19·2 %. $C_5H_{10}O_3N_2$ requires C, 41·1; H, 6·9; N, 19·2 %.) The amide-N (Sachsse) was 50·2 % of the total N. For a 3·6 % solution in water the observed rotation was $\alpha_D^{22°} + 0{\cdot}35°$, whence $[\alpha]_D^{22} = +5°$; Schulze and Trier [1912] found for their purest preparations of glutamine from plant sources $[\alpha]_D + 6°$ to $+ 7°$.

SUMMARY.

Glutamine has been isolated from an enzymic digest of gliadin, thereby providing additional evidence of the validity of the amide hypothesis, which was first demonstrated by the isolation of asparagine from edestin.

The instability of glutamine in aqueous solution, and its bearing on previous work on the nature and extent of enzymic hydrolysis of proteins are briefly discussed.

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OBITUARY NOTICES.

ALFRED CHASTON CHAPMAN.

1869–1932.

In the passing of Alfred Chaston Chapman on October 17th, in his 63rd year, we have to record the loss of one who, by reason of his striking personality no less than of his scientific attainments, occupied a conspicuous position in the chemical world, and of one who enjoyed an international reputation more particularly in relation to the industrial aspects of fermentation.

He was the son of the late A. W. Chapman, formerly of Pool-in-Wharfedale and of Leeds, and was educated at the Leeds Grammar School, proceeding later to University College, London, where he studied under Williamson, Charles Graham and Carey Foster. Here at an early age he was promoted to the post of "demonstrator of chemical technology" in Graham's laboratory, and as Graham devoted the greater part of his technical activity to matters relating to brewing, Chapman's attention became early focused in this direction, with the result that, after a short period as Graham's private assistant, he was led, under encouragement from his teacher, to establish a consulting practice of his own in connection with which for many years he conducted a private school of brewing in which subject he soon became a recognised authority.

His interests, however, rapidly extended in other directions and there stands to his credit a long list of valuable investigations on multifarious subjects. Most of his contributions to our literature were published in the *Journal of the Chemical Society*, the *Analyst* and the *Journal of the Institute of Brewing*, and have been dealt with in a longer memoir which the writer, at the request of its Council, has contributed to the *Journal of the Chemical Society*; it may suffice here to direct attention more particularly to a brief record of his contributions coming more especially within the general purview of biochemistry.

His first conspicuous effort in this direction was his classical investigation on the essential oil of hops, of which he demonstrated that the principal constituent was the sesquiterpene to which he gave the name "humulene," from which he prepared a number of derivatives; he also isolated the constituent myrcene, identical with that prepared by Power from bay oil, while minor constituents of the lower boiling-point fractions were found to be linalool and linalyl *iso*nononoate, together with some less perfectly identified bodies. Among the higher-boiling fractions he found three previously unrecognised

constituents, namely, a ketone (luparone), an unsaturated alcohol (luparenol), and a phenolic ether (luparol), and he also recognised the presence of *iso*valeric acid (*J. Chem. Soc.* 1895, 780; 1903, 505; 1928, 785 and 1304).

The nitrogenous constituents of hops were also the subject of investigation by Chapman (*J. Chem. Soc.* 1914, 1895) who, among these, identified betaine, *l*-asparagine, aspartic acid, adenine, hypoxanthine and (probably) traces of coniine. Incidentally it may be mentioned that he devoted a good deal of attention to other essential oils in the analysis of some of which he became recognised as an expert.

He also carefully investigated the changes undergone by hops during storage and the assessment of their preservative effects by biological methods, as well as by analysis; his work in this direction, as on many other problems of the brewing industry, is recorded in the volumes of the *Journal of the Institute of Brewing* ranging over a period of nearly forty years.

In 1916 a sample supposed to be cod-liver oil, which found its way to his laboratory, was found to contain nearly 90 % of a hydrocarbon oil which appeared at first sight to be the result of a gross adulteration with mineral oil; but enquiry indicated that, although spurious in the sense of not being cod-liver oil, this remarkable sample was really a genuine product of the livers of certain fish, namely, two Mediterranean species of the shark family, *Centrophorus granulosis* and *Scymnus lichia*. Chapman arranged that a supply of the livers of these fish should be submitted to him from which he extracted an abundance of oil similar to the sample originally examined, containing about 90 % of hydrocarbon oil. To this remarkable hydrocarbon he gave the name "spinacene," describing it as an optically inactive chain compound having the empirical formula $C_{30}H_{50}$ and yielding the saturated compound $C_{30}H_{62}$. By a curious coincidence, unknown to Chapman until he had presented his original paper to the Chemical Society (*J. Chem. Soc.* 1917, 56), a similar hydrocarbon had been independently isolated in Japan by M. Tsujimoto from two other fish of the same family, to which the discoverer had assigned a similar formula and for which he had proposed the name "squalene." Chapman continued his investigation of spinacene (*J. Chem. Soc.* 1918, 458 and 1923, 769), making a closer study of its physical constants and preparing further derivatives, concluding that a more correct empirical formula was $C_{29}H_{48}$, and that it bore a close relation to the terpenes and apparently to cholesterol, which was interesting in view of its origin.

Another memorable biological paper was communicated in 1926 to the Society of Public Analysts (*Analyst*, 1926, **51**, 548) on "The Presence of Compounds of Arsenic in Marine Crustaceans and Shell Fish." Chapman found in the wet flesh of oysters from 10 to more than 30 parts per million of arsenic, calculated as arsenious oxide, while (similarly calculated on the wet edible portion) he found in escallops 85 parts per million, in mussels 100 parts per million, in lobsters from 35 to 100 parts per million, in crabs 36 to 70 parts per million, in shrimps and prawns from 17 to 174 parts

per million, and similar quantities in crawfish, cockles and periwinkles. That the arsenic was in organic combination was sufficiently indicated, but the form of combination still awaits elucidation. The presence of very appreciable traces of arsenic in fish had been previously demonstrated during the work of a Swedish Government Commission and by H. E. Cox in England, and its presence in oysters had been shown in our own Government laboratory in an investigation made for the Ministry of Agriculture and Fisheries; but Chapman's results were by comparison startling, raising the question as to whether these relatively heavy quantities of arsenic, in whatsoever combination it might exist, might not have a bearing on the well-known idiosyncratic susceptibility of some people to distressing cutaneous symptoms as a sequel to the consumption of lobsters or shellfish. Fresh-water crayfish and molluscs were found to be practically free from arsenic.

Chapman was the author, in conjunction with his assistant, Mr F. G. S. Baker, of an "Atlas of the *Saccharomycetes*," published by the *Brewing Trade Review*, 1905, containing more than 100 beautifully executed photomicrographs with descriptive notes. In 1920 he delivered the Cantor lectures before the Royal Society of Arts on "Micro-organisms and some of their Industrial Uses." In these lectures he pleaded for the foundation of a National Institute for Industrial Microbiology, and further emphasised the need of this in his Presidential Address to the Royal Microscopical Society in 1926.

In addition to this presidency, he had been President of the Society of Public Analysts, of the Institute of Chemistry and of the Institute of Brewing; and he was a member of the Royal Commission on Awards to Inventors, of the Scientific Panel of the Board of Trade under the Safeguarding of Industries Act, of the Forest Products Research Board of the Department of Scientific and Industrial Research, and of the Advisory Committee on Plant and Animal Products of the Imperial Institute. The Fellowship of the Royal Society was conferred upon him in 1920, and he was an Honorary Member of the Société de Zymologie of Belgium, and an Honorary Professor of the École Supérieur de Brasserie of Ghent.

The versatility of his chemical interests and his love of mingling socially with his chemical colleagues, his general amiability and his kindly readiness in extending advice or assistance to others, will make his loss at a comparatively early age keenly felt in a wide circle of friends at home and abroad, many of whom will extend their sympathy to Mrs Chapman (the daughter of his old friend, Mr John Astleford of Bournemouth), to whom he was married in 1906 and who has for many years played a pleasant part in social chemical gatherings.

B. D.

SIDNEY JAMES BUCHANAN CONNELL.

1900–1932.

S. J. B. CONNELL, whose death occurred in South Africa in 1932, was born in 1900. He was educated at Charterhouse and won an Exhibition to University College, London, where he graduated B.Sc. in 1921. In the following year he entered Prof. Drummond's research laboratory where he worked on the form of sulphur in proteins and on the estimation of lipochromes. In 1923 he went as assistant to Dr Zilva at the Lister Institute and collaborated in two papers on the antiscorbutic vitamin published during 1924. Always delicate in health he was obliged to leave England in 1924 and took up farming in South Africa, where he remained until his death.

Connell had an original turn of mind and great ingenuity in devising apparatus for special purposes. He made many friends at University College and the Lister, by whom his early death is greatly deplored.

A. H.

MOSES JOHN ROWLANDS.

1877–1932.

M. J. ROWLANDS began his medical training at University College Hospital, London, and later studied at Brussels and Berlin. On his return to London he established a practice as a consultant which, except for a period during the War when he acted as Pathologist at Military Hospitals at Mile End and Netley, he maintained until a few years ago when he decided to devote his whole time to other interests he had built up.

His early life had given him a deep love of the country and he farmed a considerable acreage of land between London and the South Coast. To this estate he would escape whenever he could and spend his time from morning to night superintending the activities there. He became a highly successful breeder of pedigree pigs and spent a great deal of time and money in carrying out investigations on problems of practical animal feeding. His wide experience in this field gained for him a considerable reputation, and his writings and lectures never failed to arouse interest in agricultural circles. He did much to draw the attention of the farmer to the need for improving the "vitamin-balance" of rations. His interests in this direction grew so strong latterly that he retired from medical practice to devote himself to the commercial production of balanced food mixtures for animal consumption.

From his youth he had shown a forceful personality and the possession of apparently inexhaustible energy. In all that he did this amazing vigour and determination were revealed. His enthusiasm for original investigation was

real and he spent most of his scanty free time at the bench or making experiments at his farm. Much of his work has not been published, but his printed papers include valuable contributions to the study of rheumatoid arthritis and certain deficiency disorders in man and animals.

All who met him admired his amazing capacity for work and his boundless enthusiasm; those who made his circle of intimate friends knew him for an open-hearted and affectionate Celt. Many of us could see that he habitually overworked and that inevitably a breakdown would occur if he did not take reasonable care of his health. Rest he would not and early this year the warning came in serious form. For a time he took things quietly, but he could not remain inactive and he was soon back at work; within a week or two his condition became grave and on the 22nd of June he died.

J. C. D.

CCIII. THE SUGARS OF URINE.

I. DETERMINATION OF THE REDUCING SUGARS OF URINE.

By EDWARD STAUNTON WEST
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(*Received August 2nd, 1932.*)

Harding and Selby [1931] have recently published extensive observations on the output of fermentable sugar in normal urine. Their report prompts us to record some findings made in this laboratory during recent years. Our plan has been to study the daily excretion of both fermentable and non-fermentable sugar by normal individuals and with these data for comparison to investigate various factors which influence the excretion.

As will be noted from the introduction of the paper by Harding and Selby the question of a suitable method of analysis is paramount in an investigation of this kind. This is especially true when determination of non-fermentable as well as fermentable sugar is involved. Here the efficient pre-treatment of the urine to remove non-sugar reducing substances is imperative. Precipitation with mercuric nitrate-sodium bicarbonate according to Benedict and Osterberg [1918] is probably the most efficient treatment which has been reported in the past, yet this method has the disadvantage of leaving a high salt concentration in the filtrate and there is danger of adding too much bicarbonate with resulting oxidation of sugar by the mercuric ion. Treatment with Lloyd's reagent which was introduced by Folin and Berglund [1922] and which has been used in various ways by different workers, according to our experience, leaves much non-sugar reducing material. In order that maximum accuracy be obtained it is also advisable to use a sugar method capable of estimating small quantities.

Preliminary to their studies, the writers have developed a procedure of analysis for fermentable and non-fermentable sugar in urine which seems to them to represent an improvement over former methods. It is based upon three procedures. Filtrates of urine are prepared by the $HgSO_4$-$BaCO_3$ technique of West, Scharles and Peterson [1929] which has been found effective in removing interfering nitrogenous substances from biological fluids without introducing appreciable amounts of electrolytes and without loss of sugar.

The reducing substances are determined in these filtrates before and after yeast fermentation, using modified Shaffer-Hartmann copper reagents, made much more sensitive than the original due mainly to omission (or decrease) of KI. The reagents used are the outgrowth of unpublished work by Drs Shaffer and Somogyi. The suitability of such reagents for the determination of low concentrations of sugar has been shown also by Harding and Downs [1929], Harding and Van Nostrand [1930] and Harding and Selby. Yeast fermentation is carried out with washed yeast prepared according to Somogyi's [1927] directions. We believe that this combination gives more nearly correct reducing values for fermentable and non-nitrogenous non-fermentable reducing substances (possibly of carbohydrate nature), than has been achieved by other procedures.

This paper reports in detail the analytical methods used and the daily excretion of fermentable and non-fermentable sugars in the urines of a number of normal human subjects. A second paper has to do with some of the factors which influence the excretion of these substances.

Reagents.

Mercuric sulphate. A saturated solution in 10 % H_2SO_4 prepared by dissolving 27 g. $HgSO_4$ per 100 cc. of cold acid (5–10°). The solution is decanted from the basic sulphate which separates as the solution comes to room temperature.

Barium carbonate[1]. Mallinckrodt's technical precipitated $BaCO_3$, dried.

Yeast. Fleischmann's yeast washed and kept as recommended by Somogyi.

Sugar reagent. Modified Shaffer-Hartmann containing 1 g. KI per litre, kept in pyrex flasks[2].

Urine. Urine samples were preserved with a little toluene and kept in the cold room.

Procedure of analysis.

Preparation of filtrates. 10 cc. of urine are pipetted into a 500 cc. Erlenmeyer flask, together with 45 cc. of water, and 15 cc. of mercuric sulphate reagent are added from a burette. To the mixture are added 35–40 g. of $BaCO_3$ with vigorous rotation for a few seconds until most of the CO_2 has escaped. The flask is tightly closed with a rubber stopper, shaken hard and the pressure released. This is repeated until no more pressure develops. If the mixture reddens blue litmus, a little more $BaCO_3$ is added and the shaking repeated. After neutralisation the contents of the flask are poured upon a rapid filter and the filtrate (about 25 cc.) acidified with 1 large drop of conc. H_2SO_4.

[1] One or two samples of $BaCO_3$ have been found to contain something which inhibits the action of yeast. The interfering substance is not removed by H_2S in acid solution.

[2] Details of a number of improved reagents will be published shortly in a joint paper by Drs Shaffer and Somogyi. The reagent used by the writers had the following composition in g. per litre: Na_2CO_3 (anhydrous), 25·0; $NaHCO_3$, 20·0; Rochelle salt, 25·0; $CuSO_4$, $5H_2O$, 7·5; KI, 1·0; KIO_3, 20·0 cc. of *N* sol. per litre.

The filtrate, in a 300 cc. Erlenmeyer flask, is treated with H_2S for several seconds and the H_2S removed by a rapid current of moist air. The precipitated HgS and $BaSO_4$ are filtered off on a small paper and the filtrate received in a large test-tube. The funnels and tubes used must not be contaminated by previous use with solutions containing mercury salts. It is convenient to keep a supply of funnels and tubes especially for this filtration to prevent poisoning the yeast with Hg-contaminated vessels. The time required for the preparation of filtrates is only a little greater than that consumed in making Folin-Wu blood-filtrates. The filtrations proceed rapidly. The urine-filtrates so obtained represent a dilution of 1 : 7. Greater concentration cannot well be achieved. Dilutions up to 1 : 12 give quite satisfactory results when the urines themselves are not too dilute.

Determination of reducing substances. The filtrate obtained by the above method is divided into two approximately equal portions (12–14 cc. each). About 7 cc. of a 15 % suspension of washed yeast are placed in a 15 cc. pointed centrifuge-tube and the yeast centrifuged down firmly. Supernatant liquid is poured off and the walls of the tube dried with a roll of filter-paper. One portion of filtrate is added to the yeast, mixed and allowed to ferment for 10 minutes at room temperature, the yeast being occasionally stirred. After centrifuging the yeast the fermented and unfermented filtrates are analysed for reducing substances as follows. 5 cc. portions, in duplicate, are measured into 25 × 200 mm. pyrex tubes, a drop of 0·05 % aqueous phenol red added to each, and 0·5 *N* NaOH drop by drop with shaking to the red colour of the indicator. 5 cc. of copper reagent are added, the tubes covered with glass bulbs, and heated in a steady rack in an actively boiling water-bath for 15 minutes. The tubes are then cooled to about 30°, 1 cc. of a solution containing 4 % KI and 5 % potassium oxalate is added followed by 5 cc. of *N* H_2SO_4. Upon acidification the tubes are thoroughly shaken (bulbs still in place), until all Cu_2O is dissolved, and permitted to stand for 10 minutes. They are then rinsed down, including sides of covering bulbs, with a fine stream of H_2O from a wash-bottle (3–5 cc.) and titrated with 0·005 *N* thiosulphate, adding a solution of starch towards the end. Heated blanks are run on distilled water (containing phenol red). The latter does not affect the reduction and improves the end-point of the iodimetric titration for most people.

The method of calculating results will be illustrated by an example. Suppose the sugar reagent used was such that 1 cc. of 0·005 *N* thiosulphate titration was equivalent to 0·113 mg. of glucose. The total concentration of reducing substances (as glucose) in mg. per 100 cc. for a titration difference of 3·10 cc. of 0·005 *N* thiosulphate on the unfermented filtrate would be: $3{\cdot}10 \times 0{\cdot}113 \times 7 \times 20 = 49$. A titration difference of 2·0 cc. on the fermented filtrate would represent: $2{\cdot}0 \times 15{\cdot}8 = 32$ mg./100 cc. total non-fermentable reducing substances (as glucose). $49 - 32 = 17$ mg. per 100 cc. fermentable (as glucose).

Discussion of the method.

Filtrates prepared from ordinary urines by $HgSO_4$-$BaCO_3$ precipitation contain about 2–3 mg. of N per 100 cc. of filtrate so that the N content of samples for analysis amounts to 0·1–0·15 mg. Urea, which does not interfere with the sugar determinations, represents nearly all the nitrogen. Urines from high protein diets may give mercury filtrates containing many times this amount of nitrogen as a result of the great increase in urea. Such filtrates show normal values for both fermentable and non-fermentable substances. Pure urea solutions were treated with $HgSO_4$-$BaCO_3$, and the nitrogen of the filtrates determined. The precipitations were made at a volume of 70 cc. as in the case of urine, the amount of material specified being present. 2·76 mg. of N remained in the filtrate of a solution originally containing 45 mg. of urea-N, 8·26 mg. in one containing 90 mg. and 16·7 mg. in the filtrate of a solution to which 270 mg. of urea-N had been added. Creatinine, creatine and uric acid removal were tested similarly. Using 3·75 mg. of creatinine-N (10·16 mg. creatinine) no detectable nitrogen was present in the filtrate (10 cc. digested). 7·5 and 15 mg. of creatinine-N gave filtrates containing respectively 0·68 and 1·88 mg. of unprecipitated N. Of 4·23, 8·46 and 12·69 mg. of creatine-N in solution precipitated with $HgSO_4$-$BaCO_3$, 0·71, 1·94 and 4·7 mg. respectively were found in the filtrates. In a like manner 0·23 and 0·29 mg. N remained in filtrates of solutions containing originally 2 and 3·6 mg. of uric acid-N respectively. As would be expected, uric acid and creatinine are practically undetectable in $HgSO_4$-$BaCO_3$ filtrates of urine.

It is probable that mercuric sulphate and mercuric nitrate precipitations are about equally efficient as far as the removal of interfering substances is concerned, when $BaCO_3$ is used as a neutralising agent, though the $Hg(NO_3)_2$-$BaCO_3$ filtrates of urine give somewhat higher titration values than do the $HgSO_4$-$BaCO_3$ filtrates, probably owing merely to the influence of their high electrolyte concentration on the oxidising equivalence of the copper reagent. Benedict and Osterberg [1918] reported practically no nitrogen in their $Hg(NO_3)_2$-$NaHCO_3$ filtrates. We have found it impossible to determine the organic nitrogen in such filtrates. In the Kjeldahl process of digestion the nitrous acid formed from the nitrate liberates free N_2 from amino-groups and ammonium salts. We have observed complete loss of N in the analysis of urea and glycine caused by adding nitrate during the digestion. It is probable, however, that the nitrogen contents of the $Hg(NO_3)_2$ and $HgSO_4$ filtrates are of the same order. It should be pointed out that $BaCO_3$ is superior to $NaHCO_3$ as a neutralising agent for $HgSO_4$ not only because it yields filtrates of very low electrolyte content, but also because with it there is no danger of developing local alkalinity within the solution being neutralised with consequent loss of sugar as a result of oxidation by the mercuric ion.

The effectiveness of the $HgSO_4$-$BaCO_3$ treatment in removing interfering uric acid and creatinine from solution and giving filtrates which show true

sugar values was demonstrated in the following way. A mixture containing 90 mg. of urea-N, 3·75 mg. of creatinine-N, and 2 mg. of uric acid-N in a volume of 70 cc. gave, without pre-treatment, reduction equivalent to a total of 4·3 mg. of glucose. 7·0, 3·5 and 1·4 mg. of glucose were added to this mixture and precipitated at a volume of 70 cc. as in the case of urine. By analysis 7·1, 3·6 and 1·66 mg. of fermentable sugar respectively were recovered. Titrations of the fermented filtrates were identical with those of the blanks on water. As has long been known, it is essential to remove creatinine and uric acid from urine in order to obtain accurate reduction values for non-nitrogenous non-fermentable substances. $HgSO_4$-$BaCO_3$ precipitation effectively removes sulphate and phosphate, which may be found advantageous in certain analyses. Glucose added to $HgSO_4$-$BaCO_3$ filtrates is quickly and quantitatively fermented by yeast.

Sugar added to urine is quantitatively recovered by analysis of the $HgSO_4$-$BaCO_3$ filtrates.

We have used varying quantities of yeast for fermentation and failed to note absorption of non-fermentable reducing substances from $HgSO_4$-$BaCO_3$ filtrates of urine. Van Slyke and Hawkins [1929] report absorption from Lloyd's reagent filtrates which were analysed by their ferricyanide-gasometric method. Harding and Van Nostrand [1930] found no absorption from such filtrates using essentially the same sugar reagent as we have employed. The results of Van Slyke and Hawkins are perhaps due to their ferricyanide reagent oxidising yeast-absorbable substances which are unaffected by the copper reagent.

Comparison with Folin's method.

It is conceivable that yeast may remove some iodine-absorbing substance which does not reduce copper and which is not sugar, yet, by the method used, would be calculated as fermentable sugar. Accordingly, it was considered desirable to establish the presence of copper-reducing material, utilisable by yeast, by a method not involving iodimetric titration and also to secure data for a comparison of our method with another. It should be pointed out in this connection that $HgSO_4$-$BaCO_3$ filtrates do not absorb iodine from an acid solution, which in itself suggests the improbability of such an error.

Fermentable and non-fermentable reducing substances were determined on both $HgSO_4$-$BaCO_3$ and Lloyd-permutit filtrates of urine by the sensitive Shaffer-Hartmann reagent and by Folin's [1929] reagent. The Lloyd-permutit filtrates were prepared according to Folin and Svedberg [1926]. Table I summarises the data obtained on 24-hour samples of 11 normal individuals, the fermentable and non-fermentable substances being expressed as mg. per 24-hour sample (glucose reducing equivalent). The first column also includes determinations on diluted (1 : 10; 1 : 15 for very concentrated urine) untreated urines by the Shaffer-Hartmann method for comparison.

It will be observed that the average non-fermentable reducing material was 1246 mg. (as glucose) in untreated urine, 581 mg. in Lloyd-filtrates, and

Table I. *Fermentable and non-fermentable reducing substances of urine in untreated urine, $HgSO_4$-$BaCO_3$ and Lloyd-permutit filtrates, determined by Shaffer-Hartmann and Folin methods.*

Results expressed in terms of mg. glucose per 24-hour urine.

	Shaffer-Hartmann						Folin			
	Diluted untreated urine		$HgSO_4$-$BaCO_3$		Lloyd		$HgSO_4$-$BaCO_3$		Lloyd	
Sample No.	Ferm.	Non-ferm.	Ferm.	Non-ferm.	Ferm.	Non-ferm.	Ferm.	Non-ferm.	Ferm.	Non-ferm.
1	123	1895	116	370	125	800	61	362	140	425
2	72	1310	66	304	85	560	66	270	60	406
3	55	1005	73	265	80	496	46	111	50	264
4	82	1270	72	387	66	675	59	351	53	492
5	86	770	118	244	121	428	106	210	94	286
6	41	1163	89	240	84	502	91	211	72	300
7	212	722	104	250	135	545	170	148	138	327
8	85	1465	94	278	119	585	105	237	95	353
9	41	1245	91	260	73	545	39	283	47	351
10	176	1682	119	407	130	813	128	382	164	516
11	52	1180	61	202	72	443	78	144	78	278
Min.	41	722	61	202	66	428	39	111	47	264
Max.	212	1895	119	407	135	813	170	382	164	516
Av.	93	1246	91	292	99	581	86	246	90	362

292 in the $HgSO_4$-$BaCO_3$ filtrates, all by the Shaffer-Hartmann method. Lloyd treatment removed 53 % of the total non-fermentable reducing material and $HgSO_4$-$BaCO_3$ treatment removed 76 %. An interesting point is that, whereas the average difference in reduction equivalent for non-fermentable reducing substances in mercury filtrates by the Shaffer-Hartmann and Folin methods is 16 % (292–246) the difference in Lloyd filtrates is 38 % (581–362). This suggests a decided chemical difference between the reducing material not removed by Lloyd treatment but taken out by mercury and that remaining in the mercury filtrates. If the latter is essentially carbohydrate in character, the former is probably not, and Lloyd filtrates are not to be recommended in studies of the non-fermentable sugar of urine.

The average values for fermentable sugar by the Shaffer-Hartmann method were 93 mg. in untreated urine, 91 mg. in mercury filtrates and 99 mg. in Lloyd filtrates. By the Folin method the averages were 86 mg. in mercury and 90 mg. in Lloyd filtrates. The averages are rather close together but it will be noted that the individual results on mercury filtrates by the Shaffer-Hartmann method show less range of variation than those by any of the other combinations. We have found it possible to duplicate our results by this method more satisfactorily also.

Reducing substances in normal human urine.

Determinations of the daily output of fermentable and non-fermentable reducing substances have been made on a large number of medical students and laboratory workers. The maximum output of fermentable in 55 cases

was 596 mg., the minimum 23 mg. and the average 142 mg. per day calculated as glucose. The average concentration was 12 mg. per 100 cc. with an average urine volume of 1216 cc. The diet was not controlled. The amount of fermentable material varies widely yet it is probably always present. Our results agree, in general, with those of Van Slyke and Hawkins, and Harding and Selby in so far as the results can be compared. These workers analysed samples of urine taken at definite periods during the day and after the ingestion of various foods and did not report the results on 24-hour samples. Harding and Selby observed the absence of fermentable sugar from urine after short periods of starvation. By our method we have always found fermentable sugar in starvation urine though in decreased amounts. The failure of Harding and Selby to find fermentable sugar in starvation urines is probably due to the fact that they used more highly diluted urines, a smaller sample for analysis (2 cc. instead of 5 cc.), a somewhat less sensitive sugar reagent and a shorter time of heating than did the writers. To illustrate, assuming a minimum dilution of 1 : 10 as used by these workers (their maximum dilution was 1 : 20), a urine containing 6 mg./100 cc. of glucose would give a titration difference of about 0·08 cc. of 0·005 *N* thiosulphate, and if the urine dilution had been 1 : 20 the titration difference would be only 0·04 cc., very close to their normal error of titration. The same urine analysed by our procedure would give a titration difference of about 0·37 cc. of 0·005 *N* thiosulphate, nearly five times that by the procedure of Harding and Selby using a 1 : 10 dilution. Other points relating to fermentable sugar are considered in the second paper.

The advisability of using a sensitive sugar reagent in detecting the presence of fermentable sugar in urine seems to be borne out by some of our experiments. At first we used the Somogyi modification of the Shaffer-Hartmann reagent [1926] with 10 g. of KI per litre for $HgSO_4$-$BaCO_3$ filtrates. In 20 normal cases 5 showed the absence of fermentable sugar. Using exactly the same technique, with the exception that the sensitive iodide-free sugar reagent was employed, fermentable sugar was found present in the urines of 57 normal individuals in a group of 58. Many urines analysed since this series was run have shown fermentable sugar without exception, including those from fasting subjects.

The maximum output of non-fermentable substances in 58 cases, was 554 mg. (as glucose), the minimum 273 mg., and the average 395 mg. with an average urine volume of 1208 cc. The average concentration was 33 mg. per 100 cc. The results of Van Slyke and Hawkins for non-fermentable substances were much higher, probably owing to the use of Lloyd-filtrates and the more powerfully oxidising ferricyanide reagent. It should be pointed out, however, that a comparison of non-fermentable reducing substances as obtained by different sugar methods, even with the same filtrate is meaningless as long as the nature of the material and its reducing power toward the different reagents are unknown.

SUMMARY.

An improved method for the determination of the fermentable and non-fermentable sugars of urine is presented. It is based upon the preparation of $HgSO_4$-$BaCO_3$ filtrates followed by determination of fermentable and non-fermentable sugar using washed yeast and a sensitive Shaffer-Hartmann reagent.

The writers have found fermentable sugar present in almost all 24-hour samples of normal urine examined, an average of 142 mg. (as glucose) in 55 cases.

The average non-fermentable sugar (calculated as glucose) was 395 mg. in 58 24-hour samples of normal urine.

The writers are indebted to Mr Arthur Brandon for technical assistance.

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CCIV. THE SUGARS OF URINE.

II. FACTORS AFFECTING THE EXCRETION OF FERMENTABLE AND NON-FERMENTABLE SUGARS IN URINE.

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The first paper of this series by West and Peterson [1932] outlines an improved procedure for the determination of fermentable and non-fermentable sugars in urine. Although a number of workers have studied the factors which influence the excretion of these substances, the whole question is in a rather confused and unsatisfactory state. This is largely due to the fact that in much of the older work the methods of analysis were inadequate. This applies especially to the fermentation procedures used, which involved the use of unwashed yeast acting for many hours. Under such conditions it is well recognised that the yeast itself may add an amount of reducing material which may completely offset that removed by the yeast, leading to data which indicate the complete absence of fermentable sugar, or bacterial contamination may cause sugar destruction leading to values for fermentable sugar which are too high. Other sources of error lie in the incomplete removal of non-sugar reducing substances from the urine before analysis and the use of sugar methods incapable of detecting small differences.

Benedict and Osterberg [1918] using $Hg(NO_3)_2$-$NaHCO_3$ filtrates of urine and the picrate sugar method, studied the effect of diet and starvation upon the excretion of total sugar in the dog. They found this to be highest on a carbohydrate diet, appreciably decreased on a high protein diet and markedly less during starvation. They noted a maximum excretion 4–5 hours, and a minimum excretion about 15 hours, after feeding. They found the output to be independent of urine volume. These workers with Neuwirth [1918] studied both the fermentable and non-fermentable sugars in the urine of two normal men as affected by diet. They found the total excretion to be greater, in general, for a high carbohydrate than for a high protein diet, both fermentable and non-fermentable fractions being increased. Their fermentable values for all experiments on one individual averaged nearly the same as their non-fermentable, something over 500 mg. (calculated as glucose) per 24 hours. The other subject

showed less, but approximately the same proportion of fermentable and non-fermentable sugar. The large proportion of fermentable sugar was perhaps due to long periods of fermentation with unwashed yeast during which there may have been considerable destruction of sugar by bacteria. They concluded that fermentable sugar is always present in normal urine and advanced the idea of a continued "glycuresis" or passage of blood-sugar into the urine.

Folin and Berglund [1922] studied the total sugar of urine and blood after the ingestion of large quantities of various carbohydrates. In determining urine-sugar they used Lloyd-filtrates and analysed them by the method of Folin and Wu [1920]. Fermentable sugar was not determined. They noted an increase in total sugar (Lloyd-filtrates contain much non-sugar reducing material) when passing from a high protein diet to an ordinary mixed diet containing carbohydrate, but when pure sugars (glucose or its derivatives) were ingested in large amounts there was no increased output. Fructose was found to cause an increase, especially if previously heated.

Folin and Berglund concluded that there is no "glycuresis" normally of blood-sugar into the urine as stated by Benedict and that the sugars of normal urine are essentially composed of unassimilable carbohydrate substances of the food, occurring there either naturally or formed by heating.

Greenwald, Gross and Samet [1924–25] studied the effect of diet upon the excretion of urinary sugar in both the dog and man. They used $Hg(NO_3)_2$-$NaHCO_3$ filtrates and analysed them by four different methods, the two picrate methods of Benedict and Osterberg [1918; 1921], the copper method of Folin and Berglund and that of Shaffer and Hartmann [1920–21]. Fermentable sugar was not determined. From their observations they conclude that the sugars of normal urine are composed of carbohydrates assimilable with difficulty or not at all and of reducing substances (probably pentose) derived from protein of the food and from endogenous sources. They considered at least one-half of the sugars to originate from food protein and endogenous sources. Their experiments tended to show that practically no reducing material arises from the metabolism of nucleic acids or endogenous nuclear metabolism, as shown by absence of increase in urinary sugar after the ingestion of nucleic acids or as the result of turpentine abscesses in the dog. The marked effect of heated carbohydrate material (grape-nuts) in increasing the reducing material of urine was also observed. Later Greenwald, Gross and McGuire [1927] carried out other experiments and used Eagle's [1926–27] fermentation procedure which consisted of incubation with unwashed yeast for 40 minutes. They concluded that while a part of the urinary sugar may arise from heat-altered carbohydrate of food, this fraction is rather small. The opinion expressed in the former paper that pentose derived from protein constitutes a part of the sugar of urine was abandoned. Also they considered the small fraction of fermentable sugar indicated by their data probably to represent a sugar other than glucose. They suggested the idea that some of the urinary sugar may arise from bacterial action in the intestines. No experiments were reported in support of this view.

Höst [1923], using the method of Benedict and Osterberg, studied the excretion of urinary sugar by many human subjects after various diets. He determined fermentable sugar with unwashed yeast acting for 48 hours. He concluded that bread especially contributes to the sugar of urine and that glucose is not present.

The workers cited above have carried out the principal investigations relating to the effect of diet upon urinary sugar. In none of these cases have the methods of analysis employed been capable of giving an accurate estimate of the fermentable and non-fermentable fractions, and in some no attempt at determining fermentable sugar was made. In fact it was a debatable question until recent years as to whether urine contains any fermentable sugar at all. As noted above, Benedict, Osterberg and Neuwirth found considerable quantities; Shaffer and Hartmann [1920–21], using unwashed yeast, failed to observe fermentable sugar in urine, as also did Eagle [1926–27]. After Somogyi [1927] introduced the use of washed yeast for fermentation and the more sensitive sugar reagents had become available, several workers reported the presence of fermentable sugar in urine. Among these were Van Slyke and Hawkins [1929], and Peterson and West [1929]. At the present time, then, some investigators agree that normal urine contains both fermentable and non-fermentable sugar, though there still seems to be no evidence in the literature that the fermentable fraction is glucose. This represents a separate problem which will be considered in the third paper of this series by West and Steiner. Suffice it to say that evidence has been obtained from experiments on the rate of fermentation of isolated sugars of urine in the Warburg apparatus indicating that the fermentable sugar of urine is glucose.

In view of the conflicting opinions arising from this older work the writers have applied their methods of analysis, reported in the first paper, in an attempt to determine more certainly some of the factors influencing the excretion of fermentable and non-fermentable sugars in the urine. Evidently much of the work is repetition of that formerly done by the older methods of analysis. This seemed not only desirable, but necessary, in order that we might continue our studies as to the chemical nature of these sugars.

The factors specifically studied have included the effects of diet, starvation, renal damage, glandular stimulation and constipation.

Factors affecting the excretion of fermentable and non-fermentable sugars.

Diet.

Non-fermentable sugars. A diet entirely free from substances that will contribute to the non-fermentable fraction cannot be realised. Accordingly, a preliminary study was made to discover the foods which, taken in reasonable quantities, have no marked effect upon the excretion of non-fermentable substances. It was found in brief that white bread, milk, potatoes, sucrose

starch, fats, peas, string beans, tomatoes, citrus fruits, canned pineapple and pears, strawberries, bananas, lettuce, cabbage, celery, eggs, cheese, cream of wheat, meats cooked without cereal, and some other articles of food can be taken rather interchangeably in mixed diets with a practically constant elimination of non-fermentable reducing substances.

A number of experiments on different individuals in which the subjects ate mixed diets of these foods on successive days were run. The results on two individuals are illustrative. Over a period of 7 days, A.C.L. excreted daily a maximum of 360 mg. of non-fermentable sugar (as glucose), a minimum of 312 and an average of 335 mg.; a maximum of 136 mg. of fermentable sugar, a minimum of 96 and an average of 117 mg. In the case of E.S.W. over a period of 5 days a maximum of 351 mg. of non-fermentable sugar, a minimum of 312 and an average of 325 mg. were eliminated. The fermentable sugar output showed a maximum of 158 mg. and a minimum of 111 mg. The values can be made to vary appreciably from these by varying the relative proportions of the different foods taken, though it seems that significantly high values cannot be produced by quantities which the average person cares to eat. Urine volume was found to be not detectably related to the excretion of reducing substances, even with extreme diuresis induced by forced fluids. This is in accord with the observations of other workers.

Muscular exercise appears to be unrelated to the output of the non-fermentable fraction as found by experiments on a subject exercising normally and then resting in bed.

In order to determine the effect of the ingestion of large quantities of different proteins, carbohydrates, fruits and other substances, restricted diets of the above foods were employed. The subject was fed with the restricted diet for 2–3 days previous to the administration of the food or substance to be tested and also for 1–2 days thereafter if a positive effect was noted.

A. The following combinations either supplementing or replacing the restricted diet caused no significant increase and in some instances a decrease of the 24-hour excretion of non-fermentable reducing substances: thirty egg-whites; 280 g. of cheese and 12 eggs; 1000 g. of herring roe; 1125 g. of brains (pig); 900 g. of boiled kidney (beef); 900 g. of boiled liver (pig); 675 g. of pancreas (ox); 675 g. of boiled beefsteak; 560 g. of canned sweet corn (Del Monte); 200 g. of dextrin (Merck's white), 100 g. at 10.30 a.m. and 100 g. at 3.30 p.m.; 600 g. of glucose in 4 portions of 150 g. each during the day; 200 g. of starch and 250 g. of sucrose; 14 g. of yeast nucleic acid; 1200 g. of bananas (net); 1350 g. of white seedless grapes.

B. The foods listed below did cause a marked increase in the output of non-fermentable substances: 500 g. of grape-nuts, 936 mg., 125 % increase; 450 g. of blue label Karo syrup (dark), 643 mg., 68 % increase; 125 g. of caramelised sucrose, 534 mg., 68 % increase; 400 g. of honey in 3 portions, 555 mg., 83 % increase; 450 g. of raisins and 280 g. of dates, 884 mg., 115 % increase; 1350 g. of unpeeled apples, 554 mg., 40 % increase.

It will be seen that the non-fermentable sugars are increased by the ingestion of such things as grape-nuts, dark Karo syrup, honey, carmelised sugar, dates, raisins and apples, but unaffected by pure carbohydrates as starch, sucrose, dextrin and glucose. These findings are in accord with the ideas of Folin and Berglund and others that much of the reducing material of normal urine represents unassimilable foreign and altered carbohydrate substances from the diet. Non-fermentable sugars are not increased beyond the values of the restricted diets by high protein intake. It was noted, however, that meat rolled in flour and cooked so as to yield caramelised carbohydrates greatly increases these substances. In the experiments reported, this was avoided, the meat being either boiled or fried plain. The effect of liver may be large if it is fried instead of boiled, presumably owing to alteration of the carbohydrate contained in it. Nucleoprotein diets and nucleic acid (yeast) caused no definite

increase of non-fermentable sugars. These results are in agreement with the findings of Greenwald, Gross and Samet [1924–25].

Non-fermentable sugars have been determined in several of the foods used. In $HgSO_4$-$BaCO_3$ filtrates of thoroughly macerated beef kidney, liver and muscle 9, 60 and 25 mg. (as glucose) per 100 g. of tissue were found respectively. These were increased by hydrolysis of the tissues for 5 hrs. in 0·5 *N* H_2SO_4 to 282 mg. in kidney, 229 in liver and 220 in muscle per 100 g. Karo corn syrup (dark), honey and caramelised cane sugar were found to yield considerable amounts of non-fermentable reducing substances both before and after hydrolysis. Ordinary commercial sucrose also gave appreciable non-fermentable reducing materials upon hydrolysis. Human faeces from a restricted diet were found to give about 20 mg. per 100 g. (as glucose) of non-fermentable reducing substances in $HgSO_4$-$BaCO_3$ filtrates.

It seems likely that practically all foods contribute something to this non-fermentable fraction which is apparently made up of a very heterogeneous group of substances essentially non-nitrogenous, and probably chiefly carbohydrate in nature.

The mercury filtrates of urine from different diets have been hydrolysed for 5 hrs. with 0·5 *N* H_2SO_4 and fermentable and non-fermentable sugars determined. Table I records data obtained and shows that the non-fermentable

Table I. *Effect of hydrolysis for 5 hrs. with 0·5 N H_2SO_4 upon the non-fermentable and fermentable sugars of urine.*

Mercury filtrates of urine				
Non-fermentable sugar total (mg.)		Fermentable sugar total (mg.)		
Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Diet
644	620	146	1684	A.C.L. Restricted + 450 g. dark Karo syrup
316	399	142	198	E.S.W. Restricted
515	545	136	1160	E.S.W. Restricted + 125 g. of caramelised sucrose
885	862	243	256	A.C.L. Restricted + raisins and dates
274	279	68	129	E.S.W. Second day of starvation.
305	354	102	230	E.S.W. 1000 g. herring roe
936	826	72	107	E.S.W. 500 g. grape-nuts

fraction is not, on the average, appreciably increased by hydrolysis and is, for the most part, not of a polysaccharide character. Crystalline osazones (apparently a mixture) have been prepared from fermented mercury filtrates of normal urine, controls being run on the yeast. This material melted at 165–170° (uncorr.) with decomposition at 175°, and seemed to resemble the product

obtained by Patterson [1926]. It is interesting to observe that the fermentable substances in $HgSO_4$-$BaCO_3$ filtrates are appreciably increased by hydrolysis, even during starvation. We have found this to be true in a number of experiments not listed in the table. It should be noted that Folin and Berglund [1922] observed an increase in total sugar of urine after hydrolysis as did Greenwald, Gross and McGuire [1927]. These workers, however, did not determine that the increase is chiefly concerned with the fermentable fraction of the sugars. Since this apparently cannot arise from the non-fermentable fraction, the conclusion seems warranted that diffusible non-reducing polysaccharides are normally excreted. It will be pointed out in the third paper that there is probably very little if any fermentable reducing polysaccharide in urine and, bearing the above facts in mind, it seems that the carbohydrates of both normal and starvation urines may be chiefly grouped into the following three classes:

A	B	C
Non-fermentable simple sugars or derivatives	Non-reducing polysaccharide material	Fermentable sugar
Unchanged by acid hydrolysis	Converted into fermentable sugar by hydrolysis	Glucose

The kidneys must effect a relatively very high concentration of the polysaccharide substances from the blood because Scharles and West [1931] have shown the absence of detectable hydrolysable sugar from tungstic acid blood-filtrates. The same thing is true for the non-fermentable reducing fractions of mercury filtrates.

It has regularly been observed that the ingestion of large quantities of sucrose leads to increased values for fermentable sugar. This can be accounted for in part or entirely by the excretion of unchanged sucrose in the urine, some of which is inverted during the acid mercuric sulphate precipitation used in the preparation of filtrates. Sucrose solutions treated with the mercuric sulphate reagent as in the preparation of urine-filtrates have been shown to be considerably hydrolysed. Values for fermentable sugar after the ingestion of quantities of sucrose sufficient to cause sucrosuria, are, therefore, not significant when the urine is precipitated by a strongly acid reagent such as $HgSO_4$ or $Hg(NO_3)_2$.

Fermentable sugar. The state of activity of the pancreas and general ability of the body to metabolise glucose are recognised as important controlling factors in the elimination of fermentable sugar in the urine. For a discussion of this effect the papers of Greenwald, Gross and Samet [1924–25], and those of Heinbecker [1928] and Dann and Chambers [1930] may be consulted. It is generally recognised that carbohydrate starvation as induced by fasting and to a less degree by protein and fat diets decreases the ability of an animal to metabolise carbohydrates. This condition followed by a carbohydrate meal may lead to a glycosuria which is easily detectable by the usual qualitative sugar tests. This effect on the small quantity of fermentable sugar normally excreted, which would not be recognised as glycosuria by the ordinary tests, seems to be clearly shown in a series of experiments on E.S.W.

On 8. xi. 29, with a restricted diet, the fermentable sugar excretion was 159 mg. The following day the subject fasted and the fermentable sugar dropped to 62 mg. The next day 900 g. of boiled pig-liver constituted the sole diet and the fermentable fraction was 76 mg. The following day an ordinary restricted diet was taken which normally would have caused the excretion of not over 150 mg. of fermentable sugar. Actually 445 mg. were found, about three times the expected amount. The reverse effect seems to be shown in another series on the same subject. On 19. xi. 29, E.S.W. excreted 131 mg. of fermentable sugar on a restricted diet. The next day the restricted diet *plus* 100 g. of sucrose at breakfast, 50 g. at lunch and 100 g. at dinner was taken, a total of 250 g. of sucrose. The day following, a restricted diet *plus* 525 g. of sucrose was taken. The next day, a restricted diet *plus* 150 g. of glucose at breakfast, 150 g. at 10 a.m., 150 g. at lunch and 150 g. at 4.30 p.m. was taken, a total of 600 g. of glucose. The fermentable sugar excretion was 76 mg. and the following day on a restricted diet it was 65 mg. Here by high carbohydrate feeding and stimulation of the pancreas and possibly other carbohydrate-metabolising apparatus on successive days the excretion of fermentable sugar was reduced below the normal on a restricted diet.

Such experiments suggest that the very small quantity of fermentable sugar normally excreted is controlled by the factors operating in general carbohydrate metabolism and that, consequently, the sugar is glucose. Data presented in the third paper substantiate this conclusion. It seems, then, necessary to return to Benedict's view of a continuous "glycuresis" or passage of blood-sugar into the urine. We wish to emphasise, however, that the quantity of sugar passing is so small that it is not detectable by the ordinary qualitative tests and that it is of no clinical importance.

Protein diet preceded by starvation and purgation.

The object of this experiment was to correlate the rate and amount of excretion of fermentable and non-fermentable sugar with that of nitrogen elimination after a protein diet. Greenwald, Gross and McGuire [1927] studied the effect of the ingestion of a protein-fat diet upon the hourly excretion of sugar and nitrogen. They fed mainly eggs as the source of protein and observed no increase in the total sugar output. It should be noted that their total protein intake was relatively small, and also that the present writers have found the non-fermentable sugars from high egg diets quite small. In our experiments preliminary starvation and purgation were employed to remove the residues of previous meals from the intestinal tract. Subject A.C.L. fasted on 8. ii. 29, and took a dose of Epsom salts at 2 p.m. The next day urine samples were collected in 2-hour periods from 5 a.m. to 9 p.m. and 450 g. of lean beef were eaten at 9 a.m. The excretion of non-fermentable and fermentable sugars and of nitrogen for the 2-hour periods was determined. Fig. 1 shows the relations. Non-fermentable sugar was very definitely increased during the 2-hour period following the meat ingestion, while nitrogen remained at about the same level. Both non-fermentable sugar and nitrogen output reached a maximum during the second 2 hours, and fell off sharply 6 hours after the meat and gradually returned towards the basal values. The parallelism of the curves suggests that the non-fermentable sugars arise directly from substances contained in the meat or else are a result of the glandular or other activity concerned with its digestion and absorption. The promptness with which

elimination of non-fermentable sugar begins after meat feeding suggests that some of it may arise endogenously from the organs of digestion. This point has been tested in experiments on the dog from which we conclude that glandular activity does not contribute. These experiments will be given later in the discussion.

A sharp increase in fermentable sugar occurred during the third period after the meat was taken. It is probable also that the glucose of the blood reached a maximum about this time.

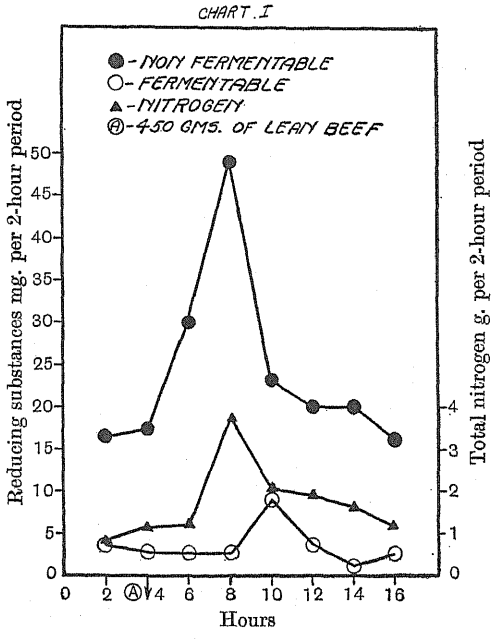


Fig. 1.

Starvation. So far as we are aware there are very few data in the literature on the effect of starvation upon the excretion of non-fermentable and fermentable sugar by human subjects. Harding and Selby [1931] have recently reported the absence of fermentable sugar from normal fasting urines within the limits of the analytical methods employed. We have pointed out in our first paper that the method used by these workers is incapable of estimating with sufficient accuracy the fermentable sugar of starvation urines. We have never failed to find fermentable sugar in the starvation urine of both human beings and the dog, although often in concentrations which the method of Harding and Selby would have failed to detect. We have made observations upon several individuals employing periods of starvation of 1 and 2 days. The results for three subjects are given in Table II. The decrease in non-fermentable sugar was rather less than we had expected and on the second day of starvation this fraction generally showed a slight rise. Fermentable sugar was continually

Table II. *Showing the effect of short periods of starvation upon the excretion of fermentable and non-fermentable sugars of urine.*

Date	Subject	Total N.F. (as glucose) mg.	Total F. (as glucose) mg.	Diet
10. vi. 29	E.S.W.	349	326	Normal
11. vi. 29	,,	265	308	Starvation
12. vi. 29	,,	274	68	Starvation. True blood-sugar 74 at 5 p.m.
16. vi. 29	H.L.W.	368	142	Normal
17. vi. 29	,,	225	38	Starvation
18. vi. 29	,,	279	160	Starvation
5. xii. 29	R.H.W.	245	—	High protein
6. xii. 29	,,	165	33	Starvation

present. The non-fermentable sugar of starvation must have come from either endogenous sources or food residues remaining in the intestinal tract or both. If food residues were the major factor, a sharp falling off in the output might be expected on the second day of starvation, especially as experiments on the rate of excretion after the ingestion of food show that non-fermentable sugar is eliminated promptly.

Experiments on the dog. In order to establish the effect of prolonged periods of starvation upon the excretion of non-fermentable and fermentable sugar a series of experiments was carried out upon the dog. A female weighing 11·3 kg. and thoroughly house-broken was selected. The animal was kept in a metabolism cage and the urine collected by catheter in 12 or 24-hour periods and preserved in the usual way. No trouble with bladder infection was experienced either on an all-meat diet or during starvation.

The dog was taken from the animal quarters and placed in the cage for several days preliminary to beginning the experiment. Determinations of the daily excretion of non-fermentable and fermentable sugars were then made for a period of 10 days on a diet of 1 lb. of lean horse meat daily in order to establish the normal output. This was followed by 11 days' starvation and then 9 days with a diet of meat as before starvation. Fig. 2 shows the daily excretion of non-fermentable and fermentable sugar throughout the 31 days of the experiment. Nitrogen excretion is also shown for the starvation period and a few days before and after. Several points of interest are brought out by this experiment. In the first place the great variation in non-fermentable sugar on a constant diet in the control period was unexpected, the range being from about 130 mg. to 230 mg. daily, much more than we have ever observed in human subjects on a carefully controlled diet. A chance observation seemed to throw some light upon this anomaly. It was noted that the animal showed much lower excretion of non-fermentable sugar when first taken from the animal quarters and placed in the cage than she did after some days in the cage on practically the same diet. It was also found that there was a rather gradual increase in the excretion upon confinement in the cage for several days with a kind of alternating high and low output but a general trend upward until an

average excretion of about 160 mg. daily was attained. These findings seem to be correlated with the general condition of constipation of the dog. When first taken from the animal quarters where life had been active and bowel movements more frequent and regular, the non-fermentable sugar excretion was low (about 110 mg.). In the cage the dog gradually became constipated so that after a time defaecation occurred only every four or five days, whereupon the non-fermentable sugar excretion increased. More evidence in this direction is to be found in Fig. 2 where it is seen that after the period of starvation was

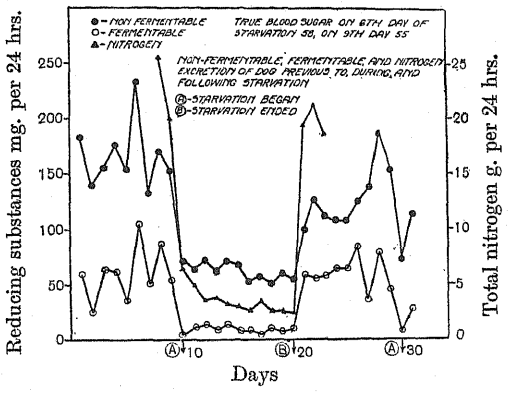


Fig. 2.

terminated, a week was required for the daily excretion of non-fermentable sugar to reach the general level of the pre-starvation period. Presumably this was about the time required to restore the normal movements of the intestinal tract.

It seems justifiable to conclude then that not only do foods contribute directly to the non-fermentable fraction, but that the processes of putrefaction and bacterial action taking place in the intestinal tract determine, in no small degree, the amount and possibly the kind of these substances excreted. These observations seem to represent the first concrete evidence presented in support of the view of Greenwald, Gross and McGuire [1927] that bacterial action in the intestine may contribute to the sugars of urine.

The output of non-fermentable sugar during the period of starvation dropped on the first day to somewhat less than half the previous level and changed very little, on the average, for 11 days. There seems to be a division into two periods, the first of 6 days and the second of 5 during each of which the average excretion was constant but somewhat higher in the first period than in the second. The constancy of the amount throughout starvation seems to suggest an endogenous origin for about a third or half of the non-fermentable sugar normally excreted. It may be that bacterial activity in the intestines produced these substances (or substance) though the constancy of the excretion would seem to preclude this action from playing more than a minor rôle.

During starvation it will be seen that fermentable sugar was continuously present in the urine though in greatly decreased amounts. Immediately after starvation the level rose to a slightly higher average, with less daily variation than before, and after about 6 days the curve resumed the shape of the pre-starvation period. The slightly higher average excretion after starvation may be a reflection of the impairment of carbohydrate metabolism by starvation.

There seems to be some alternation in the daily quantity of both non-fermentable and fermentable sugar excreted, that is, a high value followed by a low. This is not entirely regular and is disrupted for about a week after starvation when it seems to be resumed.

It is of interest to note that hydrolysis of $HgSO_4$-$BaCO_3$ filtrates of dog urine from the seventh day of starvation caused no appreciable change in the non-fermentable sugar and increased the fermentable from 12 to 22 mg. This is in general agreement with results obtained on human subjects and is possibly further evidence for the normal excretion in the urine of non-reducing polysaccharide material.

Glandular stimulation.

Attention has been directed to the promptness with which non-fermentable reducing sugar appears in the urine after taking food. This suggested that some of it may arise from glandular activity of the digestive organs and specifically from the catabolism of nucleic acids. In order to test this hypothesis experiments were made on the fasting dog in which the glands of digestion were stimulated by the sight and smell of food and by the ingestion of pilocarpine. Table III shows clearly that the excretion of both non-fermentable and fermentable sugar remained unchanged. It appears from these data then that the non-fermentable fraction of urine is unrelated to nuclear catabolism, a conclusion reached by Greenwald, Gross and Samet [1924–25] from a study of dogs with turpentine abscesses.

Table III. *Effect of stimulation of the digestive glands of the dog upon the excretion of fermentable and non-fermentable sugars.*

Date	N.F. (as glucose) mg.	F. (as glucose) mg.	Remarks
1. iii. 30	57	11	Starvation
2. iii. 30	54	10	Starvation
3. iii. 30	56	8	Starvation. Stimulated from 9·15–11·15 a.m. by placing hot meat near animal
4. iii. 30	54	10	Starvation. Injected 6 mg. of pilocarpine nitrate intramuscularly at 9 a.m. Severe salivation

Kidney impairment.

A large number of experiments have been carried out on hospital patients afflicted with various pathological conditions. The excretion of non-fermentable sugar for a number of these cases is given in Table IV. It will be noted that

Table IV. *Non-fermentable reducing sugars in the urines of hospital patients.*

No.	Diuretic	24 hour urine cc.	Non-fermentable reducing substances. Total (mg.)	Remarks
1	Salyrgan 1 cc.	1040	161	
		540	81	Patient in hospital with chronic nephritis for 20 weeks
		640	51	
	Salyrgan 0·75 cc.	640	107	Successive days
		540	38	4 days later
	Salyrgan 2 cc.	700	151	
		500	105	Successive days
		—	99	
2		900	81	Cardiac arteriosclerosis
		800	80	
3		810	154	Cardiac decompensation
	Digitalis, 2 days	3900	292	
	Digitalis	1400	266	
4		1310	203	Adult rickets
5		1250	400	Anaemia. P.S.P.* normal
6	Digitalis	2800	420	Aortitis. P.S.P. normal
7		1250	356	Scarlet fever. 8 year child. Casts, otherwise normal
8		830	0	Haemorrhagic nephritis. Non-protein N normal
		600	42	8 days later
9	Digitalis	750	382	Mitral stenosis. No arteriosclerosis. Restricted fluids
		225	166	2 days later
10		850	0	Cardiac nephritis. Non-protein N 56
11	Digitalis	760	167	Cardiac arteriosclerosis
12		1550	163	Post-hypercalcaemia
13		400	188	Diabetes, nephritis. Generalised oedema. P.S.P. 50 %
14		750	382	Determination, 15. iii. 29. Coronary infarct. Non-protein N 48, 13. iii. 29. Non-protein N 100, 18. iii. 29
14		600	324	Determination, 17. iii. 29. Died 18. iii. 29. No gross kidney lesion
Average (14, 9, 5, 6, 7 excluded)			138	
Normal—average of 58 cases			395	

* Phenolsulphonephthalein kidney test.

generally conditions leading to impaired kidney function cause a decreased output of non-fermentable sugar as would be expected. An interesting effect of the drug salyrgan[1] upon the output of non-fermentable sugar by nephritics is shown in that it causes a marked increase. It seems impossible to attribute this action to simple diuresis, because we have found, as did Harding and Van Nostrand [1930], that the output is independent of urine volume.

[1] Salyrgan is the trade name for the diuretic, sodium {*o*-[hydroxymercuric-methoxy-propylcarbamyl] phenoxy} acetate, manufactured by H. A. Metz Laboratories, Inc., New York.

By way of summary it seems that the physiological factors affecting the excretion of fermentable sugar in normal urine are quite unrelated to those controlling the excretion of the non-fermentable fraction. The fermentable sugar appears to be related to the efficiency of the general carbohydrate metabolism, and probably represents a trivial loss of blood-sugar which escapes from the normal kidney. The non-fermentable fraction, on the other hand, varies with the diet, intestinal stasis and some unknown endogenous process.

Studies relating to the chemical nature of the non-fermentable sugars of urine are planned for the future.

Summary.

A study of factors influencing the excretion of fermentable and non-fermentable sugars in urine by human beings and the dog has been made. The methods of analysis seem to offer more reliable data than have been obtained previously.

Studies on the relation of diet to the elimination of sugars have been made. Non-fermentable sugars apparently are derived in some measure from most common foodstuffs but in particular from dried fruits, apples, honey, *etc.* and carbohydrate-containing foods which have been subjected to high temperatures. This is in agreement with Folin and Berglund's [1922] conclusion that much of the reducing material of normal urine arises from altered carbohydrates of the food. Meat proteins, eggs, cheese, milk, purified carbohydrates, white bread, many fruits and common vegetables contribute somewhat, but not very much, to this fraction.

The excretion of non-fermentable sugar is not increased by high nucleoprotein diets or by the ingestion of yeast nucleic acid. Stimulation of the glands of digestion does not increase the excretion of non-fermentable sugar. Nucleoprotein metabolism seems to be entirely unrelated to this material.

In starvation experiments on both man and the dog, the excretion of non-fermentable sugar continued but at a somewhat lowered level. In the dog one-third to one-half may be endogenous and in man possibly more.

From experiments on the dog it is shown that intestinal stasis causes an increased output of non-fermentable sugar and it is concluded that bacterial action and putrefaction in the intestine may be considerable factors.

The non-fermentable sugar of urine is very little changed by hydrolysis.

Hospital patients with kidney impairment show a decreased output of non-fermentable sugar in the urine.

Fermentable sugar was found in all normal urines and in decreased amounts in the urines of persons during starvation. It was present in dog urine throughout a prolonged period of starvation.

The excretion of fermentable sugar in normal urine seems to be related to the condition of activity of the pancreas and of the general carbohydrate

metabolism. Conditions which lower the carbohydrate tolerance increase the fermentable sugar excreted and *vice versa*. This suggests that the small quantity of fermentable sugar of normal urine is glucose and that Benedict's idea of a continual passage of blood-sugar into the urine is correct. The amount present is undetectable by ordinary qualitative tests and of no clinical importance.

Evidence is presented that normally there is excreted in urine non-reducing polysaccharide material which upon hydrolysis yields fermentable sugar.

The writers are indebted to Mr George H. Curtis and Mr Arthur Brandon for aid in carrying out some of the experiments, and to Prof. Shaffer for helpful advice and criticism.

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CCV. THE SUGARS OF URINE.

III. THE CHEMICAL NATURE OF THE FERMENTABLE SUGAR OF NORMAL AND STARVATION URINE.

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That a part of the sugar of normal urine is fermentable or rather yeast-utilisable seems to have been established by the work of Van Slyke and Hawkins [1929], Peterson and West [1929], West and Peterson [1932], and West, Lange and Peterson [1932]. These last workers have also uniformly found it in starvation urines. Harding and Selby [1931] observed fermentable sugar in urines after carbohydrate and fruit diets but not in starvation urines. The failure of Harding and Selby to find fermentable sugar in starvation urines has been discussed in the second paper of this series and shown to be due probably to their analytical procedure.

The methods of analysis used by all the above workers indicate only that a part of the reducing power of urine is lost as a result of yeast action. They do not prove that a sugar, fermentable in the sense of yielding carbon dioxide and alcohol, is present. In fact, Lund and Wulf [1926] failed to observe any carbon dioxide production from normal urine when treated with pure yeast in a Barcroft manometer. These workers conclude that glucose is not a constituent of normal urine. Various investigators have prepared osazones of the urine-sugars in attempts to prove or disprove the presence of glucose. Of these Geelmuyden [1915], Höst [1923] and Patterson [1926] have reported failures to isolate *d*-phenylglucosazone from normal urines. Hassan [1928] reported the preparation of typical glucosazone crystals from many, but not all, normal urines. Greenwald, Gross and McGuire [1927] have called attention to the possibility of forming glucosazone from non-fermentable sugar derivatives of urine, especially the anhydro-sugars. Everett and Sheppard [1932] have pointed out some of the sources of error in attempts to identify glucosazone crystals among the osazones formed from urine-sugars.

It should be emphasised that neither the production of carbon dioxide under the action of yeast nor the isolation of glucosazone establishes the presence of glucose in urine. Glucose, fructose, mannose and some of the

anhydro-sugars give rise to glucosazone, and glucose, fructose, mannose and some disaccharides are fermented by yeast.

The failure of Lund and Wulf to obtain carbon dioxide production in their fermentation of normal urine may be related to two factors. They used 1 cc. of undiluted, untreated urine and added 0·5 cc. of a 1 % phosphoric acid solution to it before fermentation to adjust the p_H. Undiluted, untreated urine contains a large amount of non-sugar material which may inhibit yeast action on small quantities of sugar. Also their adjustment of p_H was uncertain, doubtless varying from sample to sample as the buffer content of the urines varied. They make no statements as to the p_H of any of their samples. Some normal urines may contain only 5–8 mg. of glucose per 100 cc. and in such cases they were working with 0·05–0·08 mg. of sugar. This quantity would produce from 11 to 17 mm.^{3} of carbon dioxide which may have been absorbed by the buffers of their solutions without registering on the manometer.

Because of the unsatisfactory state of the problem, resulting from inadequate methods, the writers have tried a different approach in an attempt to establish the presence or absence of glucose in both normal and starvation urines. It is a well-established fact that sugars are fermented at different rates and it is possible, by use of the Warburg apparatus, to obtain a characteristic curve of carbon dioxide production for each fermentable sugar. In order that such a procedure may be successfully applied to urine it is advisable that the urine be treated to remove most of the nitrogenous and other non-sugar constituents and that the p_H of the solution be accurately adjusted. Also the solution fermented should contain much more sugar than undiluted, normal urine. These demands have been met in the following way. $HgSO_4$-$BaCO_3$ filtrates of large quantities of urine (800 cc.) were prepared according to the procedure already outlined [West and Peterson, 1932]. These filtrates were evaporated to dryness, taken up in a small quantity of H_2O and precipitated with alcohol to remove salts. The alcoholic solutions were evaporated to dryness, taken up in H_2O and again precipitated with $HgSO_4$-$BaCO_3$. Evaporation of the filtrates and precipitation with alcohol were repeated and the alcoholic solution, free of salts, was evaporated to dryness. The residue was then taken up in 0·15 *M* NaH_2PO_4. By this procedure a sugar solution of the desired sugar concentration and p_H and relatively free from nitrogenous and other non-sugar substances is obtained. Suitable quantities of these solutions were fermented with washed yeast in the Warburg apparatus and the rate of carbon dioxide formation observed. Urine-sugar residues were also taken up in 0·5 *N* H_2SO_4 and hydrolysed before running fermentation curves. Solutions of glucose, fructose and mannose of the same reduction equivalents as the fermentable sugar of the urine samples were fermented in exactly the same manner simultaneously. In this way a series of curves of the fermentable hexoses was obtained under the same conditions of concentration (reduction equivalent), p_H and temperature as for the fermentable sugar of the urine samples.

Experimental.

Urine samples.

The daily fermentable sugar excretion of the three subjects who supplied urine for the following work was normal, there being no indication of a low renal threshold. For the collection of normal urine samples the subject was placed on a sugar- and fruit-free diet for 2 days, the sample being taken the second day. This was done to eliminate the possibility of sugar appearing in the urine from the ingestion of fruit juices *etc.* as found by Harding and Selby [1931]. For starvation urines the subject ate supper and began collecting the sample the next day about 15 hours after the last meal, which was sugar- and fruit-free. Urines were preserved during collection and for the short period before use by means of toluene.

Preparation of urine-sugars for fermentation.

Two portions of 400 cc. of urine are precipitated as follows and the filtrates combined.

400 cc. of urine and 1800 cc. of H_2O are placed in a 6-litre Florence flask. To this are added 600 cc. of a saturated solution of $HgSO_4$ in 10 % H_2SO_4 (about 28 g. of $HgSO_4$ per 100 cc.) with rapid mixing. Solid precipitated $BaCO_3$ is then quickly added and the solution neutralised as rapidly as possible by continual shaking and addition of further portions of $BaCO_3$. The reaction is complete when CO_2 ceases to be evolved and the solution no longer reddens blue litmus. The precipitate is filtered off on washed papers with suction, and the filtrate (4000–5000 cc.) is treated with 2 cc. of conc. H_2SO_4 per litre. Mercury is removed from the filtrate by H_2S and the latter is blown off with air. The solution is then neutralised with pure $BaCO_3$ and filtered at the pump through washed papers. A few drops of conc. H_2SO_4 are added to the filtrate and a little NaH_2PO_4 so that it reacts acid to litmus and alkaline to Congo red. A little toluene is added to the solution which is then evaporated to dryness at a temperature of 30–35° under reduced pressure, with a stream of CO_2 passing through it. At intervals the evaporation is interrupted and more NaH_2PO_4 added, if necessary, to maintain a faintly acid reaction. The residue is taken up in about 50 cc. of H_2O, precipitated with 1000 cc. of alcohol, and after standing overnight the solution is filtered and the alcohol evaporated *in vacuo* at 30–35°. The residue is taken up in 165 cc. of H_2O and precipitated with 45 cc. of the $HgSO_4$ reagent, neutralised with $BaCO_3$ and excess mercury removed with H_2S in the usual way. The filtrate is neutralised with $BaCO_3$ and the reaction made faintly acid with a drop or two of conc. H_2SO_4 and a little NaH_2PO_4 and the solution evaporated to dryness as in the first case. The residue is taken up in 25 cc. of H_2O and precipitated with 500 cc. of alcohol. After standing overnight the solution is filtered and divided into two equal parts. Both are evaporated to dryness *in vacuo* at 30–35°. One residue is taken up in 25 cc. of 0·5 *N* H_2SO_4 and hydrolysed in a boiling water-bath for 5 hours. The solution

is then diluted to 300 cc. with H_2O, neutralised with pure $BaCO_3$, filtered through washed papers at the pump, the precipitate washed with 50 cc. of H_2O and the washings combined with the filtrate. The solution is then rendered faintly acid with NaH_2PO_4 and evaporated to dryness *in vacuo* in the usual way. The residue is taken up in about 20 cc. of H_2O, precipitated with 300 cc. of alcohol, filtered, and the alcoholic solution evaporated *in vacuo* as above. The hydrolysed residue remaining is taken up in 15–20 cc. of 0·15 *M* NaH_2PO_4 as is also the other residue which has not been hydrolysed. Fermentable sugar is then determined in the solutions, after proper dilution, by the method of West and Peterson [1932]. This generally amounts to 0·5 mg. or more per cc. of solution.

In order to determine whether the fermentable sugar might arise from the filter-paper used, a water blank was run through in the same way as urine. No fermentable sugar was found either before or after hydrolysis.

The $HgSO_4$-$BaCO_3$ filtrates are kept slightly acid during evaporation, otherwise the alkalinity produced as the solutions concentrate may bring about interconversion of related sugars.

There is considerable loss of sugar in the process, probably referable to retention in the precipitates and possibly to esterification with the phosphate added during evaporation. When only one $HgSO_4$ precipitation is performed and evaporation is done without phosphate, the loss is small.

The nitrogen content of such solutions generally amounts to 0·5 mg. or less per cc. or about 10 mg. total N in the entire solution as contrasted with about 4000 mg. in the original untreated urine. It has been found desirable to use the double precipitation with $HgSO_4$-$BaCO_3$ in order more efficiently to remove non-sugar materials which interfere with and inhibit fermentation.

Fermentation in the Warburg apparatus.

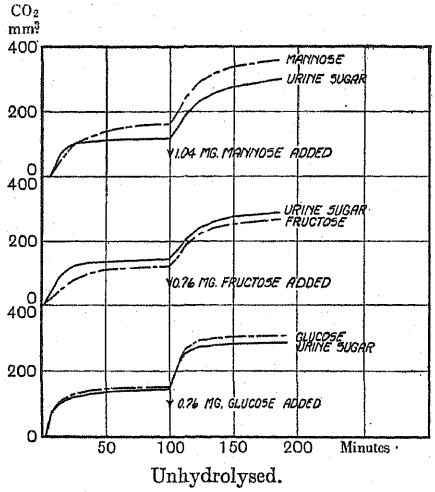
The fermentations were done in the usual way. Vessels with two side-arms were used. Three series were set up in which the urine-sugars were fermented simultaneously with glucose, fructose and mannose, all made up in 0·15 *M* NaH_2PO_4 solution. These control sugar solutions were prepared so that there was present in 0·2 cc. a quantity of sugar equivalent in reduction value to the fermentable urine-sugar. The arrangements were as follows. 1–2 cc. of the urine-sugar solution were placed in the vessel. Sufficient 0·15 *M* NaH_2PO_4 was then added to bring the volume to 3 cc. 0·2 cc. of a 20 % suspension of washed Fleischman's yeast was placed in one side-arm, and 0·2 cc. of the control sugar (glucose, fructose or mannose) in the other. The controls of glucose, fructose and mannose were prepared in the following way. 0·2 cc. of the sugar solution was placed in the vessel with 2·8 cc. of NaH_2PO_4 solution. 0·2 cc. of the sugar solution was also placed in one side-arm, and 0·2 cc. of the yeast suspension in the other. The vessels, with manometers, were then equilibrated with nitrogen and placed in the constant temperature bath at 30°. A thermobarometer containing 3·4 cc. of 0·15 *M* NaH_2PO_4 solution was placed in the bath simultaneously. After temperature equilibrium was reached the yeast

was tipped into the vessels and readings taken at 5 minute intervals throughout the period of rapid fermentation and at 10 minute intervals thereafter. Upon completion of the first fermentation the sugar solutions in the side-arm were tipped into the vessels and readings taken as before.

A fermentation was performed in which one side-arm contained saturated $Ba(OH)_2$. A large quantity of $BaCO_3$ separated indicating that the gas produced from the fermentable urine sugar was carbon dioxide.

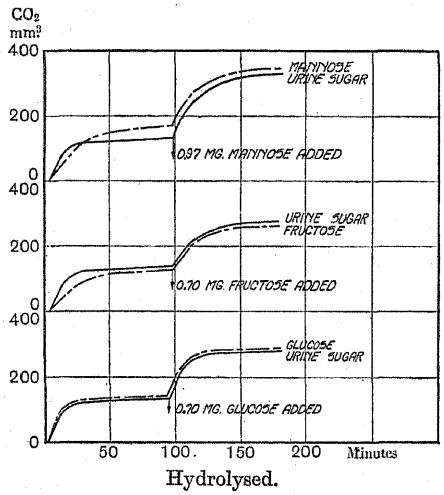
Discussion.

Figs. 1–8 summarise the results obtained on two normal urines from different subjects and similarly two starvation urines. The curves are plotted in pairs showing simultaneous fermentation of urine-sugar and an equivalent concentration (from reduction value) of glucose, fructose and mannose. The second parts of the curves represent the fermentations of sugar (equivalent to that in the original urine and control sugar solutions) added to the previously fer-



Unhydrolysed.

Fig. 1. Normal urine—I. Sugar- and fruit-free diet. Reducing power of sample = 0·758 mg. glucose.



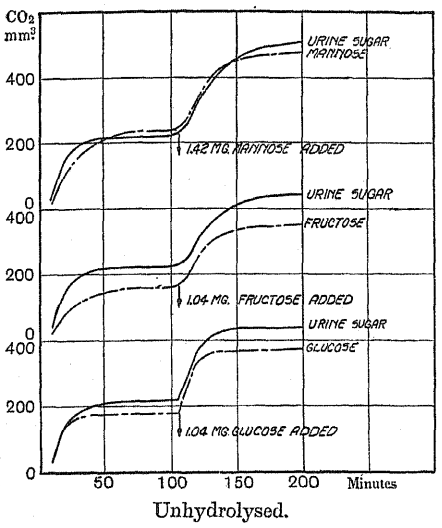
Hydrolysed.

Fig. 2. Normal urine—I. Sugar- and fruit-free diet. Reducing power of sample = 0·70 mg. glucose.

mented urine and control sugar solutions. This was done to determine whether there were non-fermentable substances in the urine samples which interfered with the rate of fermentation. It will be seen that the sugar added to the previously fermented urine-sugar solution fermented essentially similarly to that added to the fermented control.

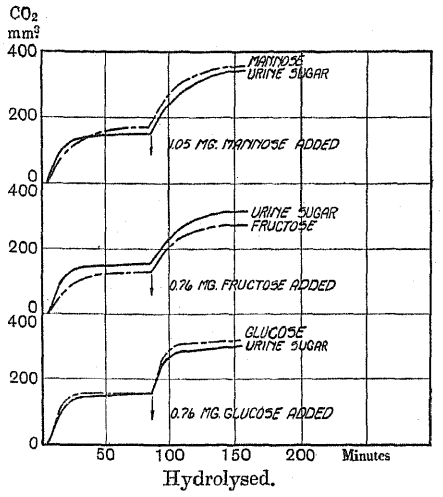
In all cases the curves of fermentation of the urine-sugar follow closely those of an equivalent quantity of glucose throughout the period of rapid fermentation, and in three cases of normal urine they run closely parallel throughout. On the other hand, the fermentation curves of fructose and mannose show entirely different rates of carbon dioxide production in all cases.

Experiments on maltose were also carried out and it was found to ferment much more slowly than the urine-sugar. We interpret these findings as evidence that



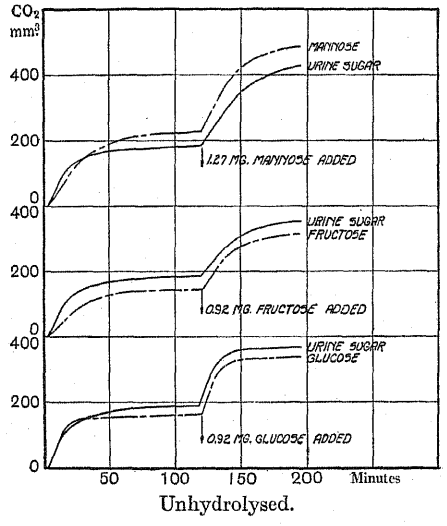
Unhydrolysed.

Fig. 3. Starvation urine—I. Reducing power of sample = 1·04 mg. glucose.



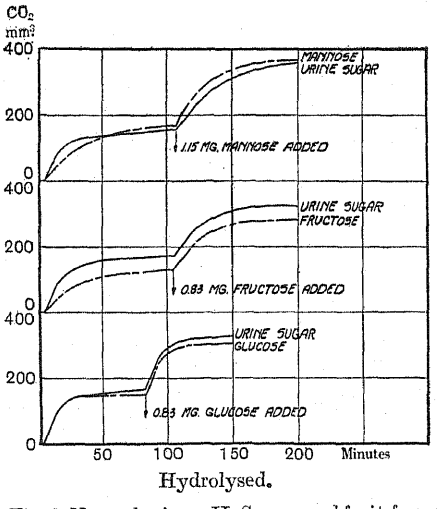
Hydrolysed.

Fig. 4. Starvation urine—I. Reducing power of sample = 0·76 mg. glucose.



Unhydrolysed.

Fig. 5. Normal urine—II. Sugar- and fruit-free diet. Reducing power of sample = 0·92 mg. glucose.



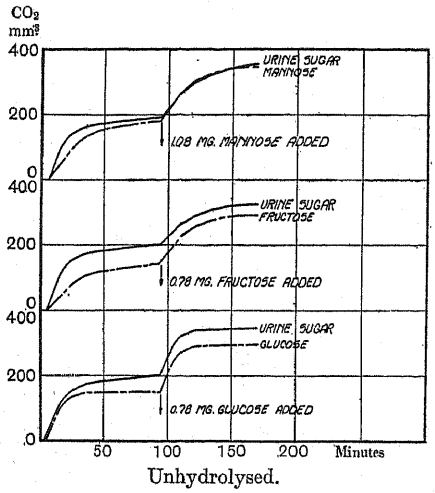
Hydrolysed.

Fig. 6. Normal urine—II. Sugar- and fruit-free diet. Reducing power of sample = 0·83 mg. glucose.

both normal and starvation urines contain fermentable sugar in the strict sense of the term and that this sugar is glucose.

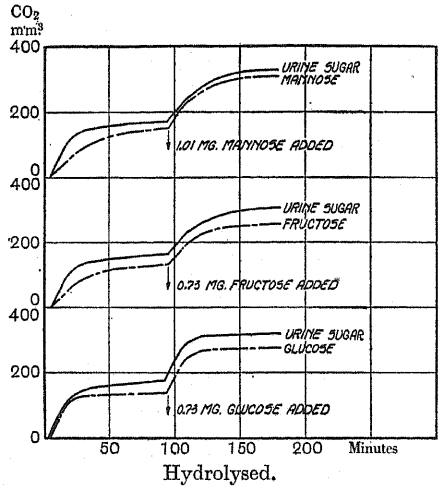
The divergence found in the final stages of fermentation in some cases may be referable to several factors. Generally the urine-sugar curve rises higher and

with a more gradual slope than the glucose curve. This may possibly be due to slow decarboxylation of an organic acid, or the slow fermentation of a disaccharide or to the stimulating effect of certain non-sugar substances upon the yeast. There may also have been slight errors in determining the fermentable sugar of the urine-sugar solutions. The slopes of the curves, however, do not indicate that this is an important factor. We have found that the addition of a little $(NH_4)_2SO_4$ or pyruvate to a glucose solution gives curves similar in form to these elevated urine-sugar curves, and are inclined to consider them, in part at least, due to the effect of such or similarly acting substances.



Unhydrolysed.

Fig. 7. Starvation urine—II. Reducing power of sample = 0·78 mg. glucose.



Hydrolysed.

Fig. 8. Starvation urine—II. Reducing power of sample = 0·73 mg. glucose.

It should be pointed out that pyruvate is not decarboxylated by yeast under the conditions of our experiments but if mixed with glucose slightly more CO_2 is evolved than from the glucose alone. The effect was removed in two cases by hydrolysis and not in another. From this it seems unlikely that it can be referred to the presence of a slowly fermentable disaccharide. Sugars of this nature would not be expected in the urines we used unless of an endogenous origin.

The glucose in urine found by us probably did not arise from the hydrolysis of anhydro-sugars referred to by Greenwald, Gross and McGuire [1927]. These should not exist in either normal urines from a sugar- and fruit-free diet, or starvation urines.

Summary.

Evidence is presented that both normal and starvation human urines contain fermentable sugar in the strict sense of the term and that this sugar is glucose. This evidence is based upon the fermentation rates of purified urine-

sugars in the Warburg apparatus simultaneously with glucose, fructose and mannose controls. The rate of carbon dioxide production from the urine-sugars parallels that from glucose but not those from mannose or fructose.

The writers are indebted to Mr R. C. Norris for technical assistance.

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CCVI. THE IMMUNOLOGICAL REACTION TO THE ENZYME PAPAIN.

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Reports have been highly contradictory with respect to the possible development of increased anti-enzymic activity in the serum of a subject which has received repeated injections of a given enzyme preparation. Wells [1925], in reviewing the subject, considers the general thesis very much open to doubt. Probably the most definite evidence of a positive nature is the work by Kirk and Sumner [1931; 1932] with crystallised urease preparations. This particular enzyme is much more stable in the presence of serum than most enzymes and such a property would seem to be a requisite for the production of an anti-enzyme. Northrop [1930], for instance, failing to obtain anti-enzymic effects with a crystallised pepsin preparation, attributed the failure to the recognised instability of pepsin in blood-serum. The vegetable enzyme, papain, by virtue of its relative stability in serum [Walton, 1931, 1, 2] is of special interest in this regard. In the previous studies [Ehrenreich, 1900; Stenitzer, 1908; Achalme, 1901; Pozerski, 1909] concerning the possible development of an antipapain effect proteolytic assay methods have been used which were evidently of slight sensitiveness. For instance, the presence of fluctuating amounts of serum-proteases in the same animal was not recognised in such studies. Ehrenreich [1900] and Achalme [1901] were unable to obtain antipapain effects with rabbits and guinea-pigs, while Stenitzer [1908] reported an anti-enzymic effect in one case with a goat. On the basis of these considerations, the problem was reinvestigated, as reported here, using a method of proteolytic assay which is unusually accurate at relatively high dilutions. The sensitiveness of this particular method was such that the development of a relatively slight anti-papain effect should have manifested itself in the recorded measurements.

Procedure.

Two healthy dogs of 8 and 10 kg. weight were each subjected to 20 intravenous and 4 intraperitoneal injections of a papain preparation during a period of 97 days. Venous blood was withdrawn at intervals and the serum used in an assay of its effect on the proteolytic activity of a solution of the papain. Samples were always taken before any injections given the same day. Intravenous

injections were borne without apparent immediate effects, while intraperitoneal injections caused considerable discomfort for a few minutes. During the course of the injections the animals lost weight and developed hemorrhagic skin manifestations, but maintained voracious appetites. On discontinuance of the injections they returned to normal.

Sterile papain for injection was obtained by pressure filtration of glycerol-water solutions through Berkefeld candles and preservation of the filtrates in sealed ampoules. Fresh filtrates, prepared about every 3 weeks, were sufficiently stable, as judged by measurements in this work and by a previous standardisation study of the same procedure [Walton, 1931, 1, 2; 1932]. The stock preparations of Parke Davis papain, whose activity has been defined in this same study, were used throughout. Injections of 1 cc. of filtrate (activity equal to about 40 mg. dry powder) are indicated in Fig. 1 by a single arrow and injections of 2 and 3 cc. by double and triple arrows, respectively.

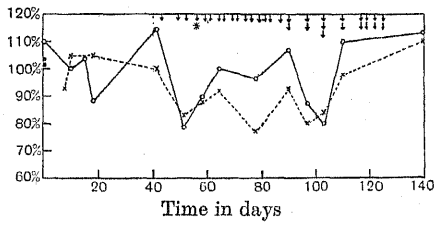


Fig. 1. Ordinates: percentage original activity of a 1–1000 papain solution after being mixed with serum (10 %).

↓ Injection of a papain solution equal in activity to 40 mg. dry powder.
■ Observations on normal dogs.

The determinations of proteolytic activity were carried out essentially according to the photographic film method as first described by Gates [1930] and by Gilman and Cowgill [1930]. The technique previously detailed by one of us [Walton, 1931, 1, 2; 1932] was modified in that no fixed temperature was used, but rather any constant temperature in the neighbourhood of 30° which would carry the standard digestion solutions (1–1000 papain) to the end-point (50 % digestion of the gelatin film) in about 20 minutes. Standards and unknowns were always run together at the same temperature. The series of digestion mixtures were sufficient in number to ensure an accuracy of ± 5 %. Papain at a 1–1000 concentration in a solution of 1 % sodium citrate was compared in its digestive activity with solutions of papain at similar concentrations but containing 10 % serum. If the serum was completely inert in this system, its 10 % mixture with a standard solution would result in a solution exhibiting 90 % of the activity of the standard solution. Observations with normal serum demonstrate the frequent presence of proteases in sufficient strength to manifest activities as high as 115 % of the standard in mixtures of this sort.

Possible serum changes due to varying intervals between time of sample taking and time of assay were shown to be without significance. Samples of

the two sera assayed at 1½ and 5 hour intervals after withdrawal showed no variation outside the accepted experimental error. These and all samples used in the study were kept on ice during this interval. A more drastic test of this point was made by heating the two sera for 1 hour at 60° before mixing with papain. Whereas the mixtures of papain with unheated sera exhibited activities of 100 % and 110 %, the mixtures with the heated sera exhibited activities of 85 % and 100 %, respectively.

Varying intervals between the time at which the serum was mixed with papain and the time of assaying the mixture were likewise shown to be without significance. Samples of the two sera mixed with papain and assayed immediately manifested substantially the same effect when assayed after the mixture had stood for 1 hour at 25°.

One comparison of the two sera taken before and after feeding showed a decrease in the proteolytic activities of the mixtures after feeding. Sera obtained before feeding exhibited activities of 85 % and 110 % in the usual mixtures with papain, while those obtained after feeding exhibited activities of 77 % and 95 %, respectively. During the course of the study, samples were taken at a regular period about 4 hours after feeding, and, as these figures indicate, some such routine procedure is essential.

Results.

Results are illustrated in Fig. 1. The normal fluctuations of serum-proteases are evidently very appreciable and any study of this type must account for such, either by subtracting the protease effect of the serum at each determination or by obtaining a sufficient number of normal determinations to establish an average. It is apparent from the general direction of the curves that the development of an antipapain effect, if present at all, is very transitory and of slight magnitude. The occasional drop in the curves may as well be due to a disturbed physiology as to an immunological reaction.

A violent reaction which occurred in both dogs on the fourth injection was of an anaphylactic nature in every observable characteristic except for the unusually brief incubation time. No further reactions of this sort occurred, although some of the subsequent injections were 3 times the dosage of that which provoked the reaction.

The sera gave precipitin reactions with filtered solutions of the papain. However, observations of this sort are complicated by the fact that normal sera gave apparent precipitin reactions with papain solutions.

Towards the latter part of the study, serum-papain mixtures of the proportions previously described were assayed before and after centrifuging off the precipitate obtained by their mixture. After removing the precipitates, which were of very appreciable bulk, the supernatant fluids exhibited substantially the same proteolytic activity as the uncentrifuged portions of the same mixture. Apparently, the proteolytic component of the papain preparations is not subject to such precipitation.

SUMMARY.

A series of observations has demonstrated the absence of any consistent tendency toward development of an anti-enzymic agent on continued injection of papain. Factors influencing the determinations of such possible effects are evaluated.

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CCVII. THE EFFECT ON SOLUTIONS OF DIPHTHERIA TOXIN AND DIPHTHERIA ANTITOXIN OF CONTACT WITH CERTAIN SURFACES.

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In some preliminary experiments on the stability of diphtheria toxin we had observed that when a dilute solution of diphtheria toxin is allowed to stand in a cylindrical glass vessel, the rate of disappearance of toxicity is greater the smaller the volume of the solution. This phenomenon probably depends, in part at least, on the greater ratio of air surface to volume of the smaller volume of liquid[1], and may depend in part on the greater ratio of glass surface to volume.

The original purpose of the work reported here was the investigation of the effect of glass surfaces on diphtheria toxin.

In order to obtain a relatively large ratio of glass surface to volume of liquid, the following arrangement was used. Pairs of pyrex glass test-tubes were selected such that for each pair one tube fitted rather snugly into the other. The outer tubes were approximately 15 cm. in length by 1·8 cm. in diameter, and the inner tubes 13 cm. in length by 1·5 cm. in diameter; the volume of liquid contained between the two glass surfaces was not greater than 2 cc. With such fitted tubes it was possible to have a ratio of surface to volume in the outer compartment at least 20 times as great as in the inner tube.

Before the tubes were used they were allowed to stand in hot sodium chromate-sulphuric acid solution for at least 2 hours, then rinsed several times in tap-water and finally in distilled water.

To carry out a test with such tubes, the inner tube of a pair was removed, 10 cc. of toxin measured in a pipette and about 2·5 cc. expelled into the outer tube. The inner tube was then replaced and pushed to the bottom, thus expelling excess liquid into the inner tube. The remainder of the toxin in the pipette was then run in. The tubes containing the toxin were kept at room temperature for about 16 hours, after which the contents of the inner and outer compart-

[1] Glenny *et al.* [1925] have shown that dilute solutions of diphtheria toxin containing phenol lose in toxicity when shaken; and that this loss of toxicity can be ascribed to the detoxifying action of phenol, the concentration of which is markedly increased in the foam [Pope, 1927]. The toxin solutions used in the experiments recorded in this paper contained no added preservative. Such solutions are also unstable to shaking.

ments were tested for toxicity by intradermal injection into normal guinea-pigs.

In these experiments it was desirable, on account of the possibility of bacterial contamination, to use aseptic technique. To this end the dry fitted tubes were plugged with cotton-wool (non-absorbent) and sterilised by heating in an air-oven at 175–185° for 1 hour. When dilute diphtheria toxin solution was put in the inner and outer compartments of the sterilised tubes, and kept at room temperature for 16 hours, it was found that the toxicity of the solutions in the outer compartments had decreased markedly as compared with those in the inner compartments. When, however, this experiment was carried out using tubes which during sterilisation had been covered with aluminium caps in place of cotton plugs, no such marked difference in the toxicity of the contents of the two compartments was observed.

A series of experiments was then carried out to determine the effect of a variety of treatments on the tubes prior to the addition of the toxin. Tubes were heated in the air-oven at 175°–185° for 1 hour—some uncovered, some covered with aluminium caps, some plugged with cotton-wool (non-absorbent), some with a small piece of cotton-wool at the bottom of the outer and the inner tubes in addition to cotton plugs, some plugged with filter-paper and some with a piece of filter-paper in the bottom of the outer and inner tubes in addition to the paper plugs. Other tubes were coated with a film of paraffin wax.

The diluted toxin which was put into these tubes was made by diluting a sterile toxic broth filtrate (80,000 M.R.D. per 0·1 cc.) with buffered saline at p_H 7·02 (1–4 dilution of McIlvaine's phosphate buffer, sodium chloride added to a concentration of 0·85 %).

Table I. *Disappearance of toxin in fitted tubes.*

Treatment of tubes prior to addition of toxin	1/4000 diln. toxin No. 461		1/2000 diln. toxin No. 461	
	Large surface / volume	Small surface / volume	Large surface / volume	Small surface / volume
Heating with no covering	—	—	19, 19, 19, 21, 17, 20	19, 20, 24, 21, 20, 21
Heated with metal caps	15, 16	19, 20	21, 21, 21	21, 21, 19
Heated with cotton plug in top and cotton at bottom of both compartments	0, 0	19, 18	0, 0, 0, 0, 0, 0, 0, 0	20, 25, 20, 20, 20, 24, 21, 22
Heated with cotton plug in top only	0, 0	20, 19	14, 12, 13F, 11, 0	24, 23, 23, 25, 23
Heated with ash-free filter-paper over top and at bottom of both compartments	—	—	0, 0, 0, 0	20, 23, 21, 24
Heated with ash-free filter-paper over top only	—	—	9F, 15, 11F	23, 23, 21
Tubes coated inside and out with washed paraffin wax	0, 0, 0	16, 16, 18	0, 0, 0, 0, 0, 0, 0, 0	19, 19, 20, 20, 22, 21, 24, 20

Note. The numbers in all the tables represent the average diameter in mm. of intradermal reactions on guinea-pigs 48 hours after injection. The experiments were set up at least in duplicate; each number represents a test on material from an individual compartment.

F=faint reaction.

After the diluted toxin had been added to the tubes, these were kept for 16 hours at room temperature. The contents were then tested by injecting 0·1 cc. amounts of the solutions intradermally into guinea-pigs. The results are summarised in Table I.

It will be observed that there is evidence of detoxification only in the case of toxin solutions from the outer compartments—large surface to volume; and further, that marked detoxification has taken place only in the tubes coated with paraffin wax and in the tubes heated with cotton and with paper. The results with the cotton and with the paper indicate that during sterilisation some volatile substance from cotton-wool or filter-paper is deposited on the glass and that this new surface is much more effective in detoxifying diphtheria toxin than is clean glass.

The conditions under which glass tubes plugged with cotton-wool are heated, prior to the addition of toxin solution, affects the capacity of the glass surface for bringing about the disappearance of toxin. This is shown in the results given in Table II. Before the tubes were heated under the various conditions indicated, a piece of cotton-wool was put into the inner and outer compartments of each pair of tubes, in addition to a cotton plug in the top. The cotton was removed from the inner and outer compartments before toxin solution was added.

Table II. *Disappearance of toxin in fitted tubes previously heated with cotton-wool at various temperatures.*

		1/1000 diln. toxin No. 461		1/2000 diln. toxin No. 461	
		Large surface / volume	Small surface / volume	Large surface / volume	Small surface / volume
Temperature	Time of heating				
78°–80° hot air	1½ hrs.	20, 17, 0	23, 22, 24	15, 16, 0	22, 21, 23
132°–133° hot air	1½ hrs.	0, 15, 11	21, 20, 20	0, 0, 0	20, 18, 20
165°–185° hot air	1½ hrs.	0, 0, 0	20, 22, 21	0, 0, 0	22, 18, 20
		0, 0, 0	24, 27, 20		
195°–218° hot air	1½ hrs.	0, 12, 0	21, 19, 20		
Autoclaved at 16 lbs. pressure	30 mins.	14, 0, 16	20, 20, 20	0, 0, 0	17, 20, 20

The maximum disappearance of toxin was obtained in tubes which had been heated at 165°–185°.

That cotton-wool itself is not effective in bringing about the disappearance of toxin is shown in Table III.

Table III. *Effect of cotton-wool on diluted diphtheria toxin.*

	Size of reaction
Unheated cotton	19
Cotton heated at 175°–185° for 1½ hrs.	17
Cotton heated at 200°–218° for 1½ hrs.	18
Cotton heated in autoclave at 16 lbs. pressure for 30 mins.	19
Toxin solution control (not in contact with cotton-wool)	19

The cotton-wool used, both heated and unheated, was placed in test-tubes and just sufficient diluted toxin added to cover it. When the tubes had stood overnight at room temperature the toxin solutions were tested by intradermal injection into guinea-pigs.

The detoxifying capacity of glass is markedly increased after contact with light petroleum[1]. This is shown in Table IV. The following experimental details concern this Table. Clean fitted tubes, covered with metal caps, were heated for 1 hour at 175–185°. A number of organic liquids—light petroleum, ethyl

Table IV. *Effect of certain organic liquids on the detoxifying capacity of glass.*

	1/2000 dilution toxin No. 461	
	Large surface / volume	Small surface / volume
Clean tubes covered with metal caps and heated in hot air-oven at 180°	24, 20, 21	20, 19, 22
Tubes treated with light petroleum and rinsed	0, 0, 0,	20, 20, 20,
	0, 0, 0,	21, 25, 20,
	0, 0, 0	19, 20, 18
,, ethyl ether ,,	18, 16	20, 18
,, toluene ,,	17, 15, 19	21, 20, 18
,, xylene ,,	17, 15, 13	17, 21, 22
,, benzene ,,	18, 13, 16	24, 25, 22
,, ethyl alcohol ,,	0, 16	21, 24

ether, *etc.*—were then put into the inner and outer compartments of the fitted tubes, the tubes stoppered with corks and left for 16 hours at room temperature. The organic liquids were then removed and the tubes rinsed 15 times with water. After this a dilute solution of toxin was put in the inner and outer compartment of each pair and the tubes left at room temperature for a further period of 16 hours. The contents were then tested for toxicity by intradermal injection into guinea-pigs.

Detoxification of dilute solutions of diphtheria toxin in fitted tubes may be prevented either by the addition of a solution of normal horse serum[2] to the toxin solution, or by first treating the tubes with dilute serum and subsequent rinsing.

The results in Tables V and VI show this protective action of serum.

A number of possibilities suggest themselves which might account for the disappearance of diphtheria toxin under the conditions which have been described above.

(1) An increase in alkalinity of the solution in the outer compartment, due to the large glass surface, might bring about a decrease in toxicity since toxin is less stable in alkaline than in neutral solution [Moloney and Taylor, 1930].

[1] Up to the moment we have not concerned ourselves with the constituent or constituents in the light petroleum used, which may be responsible for the effect shown in Table IV.

[2] Gelatin [Moloney and Taylor, 1932] is also an effective protecting agent; and no doubt many other proteins exert the same action.

Table V. *Stabilising effect of serum on toxin in tubes coated with paraffin wax.*

No serum		1/27,000 diln. serum		1/9000 diln. serum		1/3000 diln. serum		1/1000 diln. serum	
Large surface / volume	Small surface / volume	Large surface / volume	Small surface / volume	Large surface / volume	Small surface / volume	Large surface / volume	Small surface / volume	Large surface / volume	Small surface / volume
0	20	17	20	19	19	19	21	17	18
0	20	0	20	19	19	21	21	16	20
0	20	13	19	17	19	18	17	19	20

Note. The same effect is observed when glass tubes sterilised with cotton plugs are used in place of tubes coated with paraffin wax.

Table VI. *Effect of previous treatment of fitted tubes with serum on the disappearance of toxin.*

Treatment of tubes	1/2000 diln. toxin No. 461	
	Large surface / volume	Small surface / volume
Tubes sterilised with metal caps	19	17
	18	18
Tubes sterilised with cotton plugs	0	18
	0	19
	0	19
Tubes sterilised with metal caps; treated with buffer 20 hrs.; rinsed before adding toxin	22	22
	20	20
	21	21
Tubes sterilised with cotton plugs; treated with buffer 20 hrs.; rinsed 10 times before addition of toxin	16, 0, 0, 8 *v.f.* 0, 0	21, 20, 20, 23, 20, 20
Tubes sterilised with cotton plugs; treated with 1/500 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	18	20
	19	19
	19	21
Tubes sterilised with cotton plugs; treated with 1/8000 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	22	22
	20	20
	17	25
Tubes sterilised with cotton plugs; treated with 1/16,000 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	0	20
	10	21
	0	22
Tubes sterilised with cotton plugs; treated with 1/32,000 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	0	20
	0	22
	0	22
Paraffined tubes treated with 1/500 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	19	27
	22	27
	17	23

Note. Tests made on the final rinse waters showed that insufficient serum was left in the tubes to exert any protecting action.
v.f. = very faint reaction.

However, measurements made with the hydrogen electrode failed to show any difference in the p_H of solutions in the two compartments of the fitted tubes.

(2) In another communication [Moloney and Taylor, 1930] we have shown that diphtheria toxin may be rendered atoxic by means of oxidising agents. It

is conceivable that the toxin might have been destroyed by oxidation at the glass surface, owing, for example, to adsorbed oxygen or chromic acid. That oxidation is not a probable factor is shown by the following experiment. Dilute toxin solution containing sodium thiosulphate was put into fitted tubes. Examination of the solutions after the tubes had stood for 16 hours at room temperature showed a marked decrease in toxicity of those solutions which had been in contact with the large surface. Sodium thiosulphate was still present in the solutions at the conclusion of the experiment.

(3) Some detoxifying substance might be leached from the surface by the toxin solution. In connection with this possibility we have attempted to leach a detoxifying substance from glass surfaces—previously heated with cotton-wool—using as leaching agents buffered saline and saline to which serum had been added in amount insufficient to prevent the disappearance of toxin in fitted tubes. After some hours' contact with the surface, the leach solutions were used to dilute toxin and the diluted toxin was allowed to stand for 16 hours at room temperature. There was no measurable loss of the toxicity of these solutions. Moreover the tubes which had been leached had approximately the same detoxifying capacity as the untreated tubes.

(4) Toxin might be adsorbed from solution in contact with the relatively large surface. There are three ways in which adsorption might be demonstrated.

(*a*) By showing directly, with the use of animals, that the adsorption complex contains either toxin or immunising substance. This was done by Freund [1930] with diphtheria toxin adsorbed on collodion particles. In our experiments we could not conveniently carry out such a test.

(*b*) By leaching toxin from the surface. It has been shown that toxin adsorbed on aluminium hydroxide [Schmidt, 1931], magnesium hydroxide [Ecker and Wied, 1932], and barium sulphate [Siebenmann] may be recovered by suitable leaching agents. We have attempted to recover toxin from glass and paraffined glass surfaces, which had previously been treated with toxin, by the use of serum solution. There was no evidence that any toxin had been leached off.

(*c*) By showing that the surface after contact with toxin possesses specific combining power for diphtheria antitoxin. Freund [1930] and Eisler [1923] have shown that, with collodion and charcoal respectively, adsorbed diphtheria toxin will combine specifically with antitoxin. In Table VII are summarised the results of experiments in which fitted tubes sterilised with cotton plugs were first treated with a solution of diphtheria toxin, purified by the method of Siebenmann, and then tested for combining power with diphtheria antitoxin.

In these experiments there is definite evidence that diphtheria toxin has been adsorbed. It must be pointed out, however, that this holds only in the case of tubes which had been treated with solutions of purified toxin. Further, the amount of toxin which can be accounted for on the surface by antitoxin-combining power is of the order of 1/500 of the amount which will disappear from a solution in contact with an equivalent area of glass or paraffin wax

Table VII. *Antitoxin-combining power of surface after treatment with toxin.*

Method of treating tubes	Antitoxin-combining power of final rinsing	Concentration of antitoxin and serum added to tubes; 16 hrs. room temperature	Tests on antitoxic solns. from large $\frac{\text{surface}}{\text{volume}}$ compartments
Soln. purified toxin 3 Lf per cc. left in tubes for 20 hrs. at room temp. Rinsed 10 times before addition of antitoxin	Final rinse water (buffered saline) left in tubes for 3 hrs. No evidence of combining power	1/1000 unit per cc. +1/2000 normal serum	Injected 0·2 cc. of equal amounts of a toxin dilution and antitoxic solution 19 15 15 19 16 21
Fitted tubes not treated with toxin		1/1000 unit per cc. +1/2000 normal serum	0 0 10 *v.f.* 12 *v.f.*

Note. A toxic reaction denotes a decrease in concentration of antitoxin in the solutions tested. *v.f.* = very faint reaction.

surface. In other experiments we have attempted without success to demonstrate antitoxin-combining power of surfaces which had been treated with dilutions of unpurified toxic broth filtrate. None of these was successful.

This failure to demonstrate the specific adsorption of antitoxin by glass previously treated with a solution of unpurified toxin might have been due to the relatively small amount of toxin adsorbed in comparison with the amount of substances other than toxin adsorbed from the toxin broth filtrate.

Disappearance of antitoxin in contact with large surfaces.

In the experiments recorded in Table VII it was necessary to know the effect on solutions of antitoxin of contact with relatively large surfaces. Results in Table VIII show that antitoxin disappears from solution in the outer compartment of the fitted tubes—large surface to volume—which had been

Table VIII. *Disappearance of antitoxin in fitted tubes and protective action of added serum.*

	Antitoxin 1/100 unit per cc.		Antitoxin 1/100 unit per cc. +1/2000 diln. normal serum		Antitoxin 1/200 unit per cc.		Antitoxin 1/200 unit per cc. +1/2000 diln. normal serum	
Treatment of tubes	Large surface/vol. L+/200 tox.	Small surface/vol. L+/200 tox.	Large surface/vol. L+/200 tox.	Small surface/vol. L+/200 tox.	Large surface/vol. L+/300 tox.	Small surface/vol. L+/300 tox.	Large surface/vol. L+/300 tox.	Small surface/vol. L+/300 tox.
Tubes sterilised with metal caps	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
Tubes sterilised with cotton plugs	15	0	0	0	15	0	0	0
	17	0	0	0	13	0	0	0
	12	0	0	0	9	0	0	0
	16	0	0	0	—	—	—	—
	18	0	0	0	—	—	—	—
Tubes coated with paraffin wax	13	0	0	0	13	0	0	0
	15	0	0	0	13	0	0	0
	16	0	0	0	12	0	0	0

Note. The antitoxin solutions used were dilutions in buffered saline (p_H 7·02) of antitoxic horse serum 350 units per cc. which contained no added preservative.

sterilised with cotton plugs or which had been coated with paraffin wax, and that this disappearance is prevented when sufficient serum is first added to the antitoxin solution.

Conclusions.

1. Diphtheria toxin disappears from solution in contact with a sufficiently large surface of paraffin wax, or of glass which has been first in contact with light petroleum, or of glass which has been heated in an air-oven in close proximity to cotton-wool or filter-paper for one hour at 180° (tubes plugged with cotton-wool or with filter-paper). Glass, cleaned with chromate-sulphuric acid solution and heated in the air-oven at 180° for one hour, has relatively little effect on toxin solutions in contact with it.

2. Similarly diphtheria antitoxin disappears from solution in contact with a paraffin wax surface or with glass previously exposed to heated cotton-wool.

3. It is probable that the disappearance of toxin and antitoxin from solutions in contact with paraffin wax and glass is due to adsorption on the solid surfaces.

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CCVIII. A CELLULASE FROM THE SYMBIOTIC INTESTINAL FLAGELLATES OF TERMITES AND OF THE ROACH, *CRYPTOCERCUS PUNCTULATUS*.

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The number of organisms able to utilise cellulose as the sole source of carbohydrate is small, and the number from which it has been possible to isolate a cellulase is smaller still. Karrer *et al.* [1923; 1924; 1925] have done extensive work with a cellulase from the intestinal juice of the vineyard snail, as have Pringsheim and Bauer [1928] and Pringsheim and Leibowitz [1928] with a very similar enzyme obtained from malt. Boynton, Lyman and Miller [1927] were able to demonstrate cellulase somewhere in the anterior portion of the shipworm, *Bankia setacea*. Jewell and Lewis [1918] found lichenase in the alimentary system of each of twenty species of invertebrates. Since lichenin is a form of cellulose [Karrer, Joos and Staub, 1923] the active extracts from these animals would probably also have hydrolysed cellulose. Pringsheim [1912; 1919] showed long ago the presence of a cellulase in cellulose-fermenting bacteria, but this enzyme loses its activity very soon after the death of the bacteria, and has never been obtained in a cell-free condition.

Of all the cellulose-feeding organisms there are few whose diet contains as high a percentage of cellulose as does that of termites. Cleveland [1923; 1924] has shown that these insects can live for long periods of time on a diet of almost pure cellulose, if, and only if, their normal complex intestinal fauna has not been removed. This fauna consists mainly of representatives of two orders of flagellates: the polymastigotes and the hypermastigotes. The great majority of these intestinal protozoa ingest cellulose particles and apparently digest them. They might be presumed to contain a cellulase.

Most termites, on account of their small size, do not furnish very good material for the preparation of enzyme extracts. Recently, however, it has been discovered that the large wood-feeding roach, *Cryptocercus punctulatus*, possesses a fauna of intestinal flagellate symbionts very similar in all respects to those of termites [Cleveland, 1930]. These roaches, obtained through the kindness of Dr L. R. Cleveland, proved to be an excellent source of the enzyme, cellulase, and they were used for most of the work reported in this paper. The

presence of the enzyme has also been demonstrated in the termites, *Reticulitermes flavipes* and *Termopsis angusticollis*, as well as in pure cultures of one of the protozoa from the latter.

Action of the cellulase. Proof that it is secreted by the intestinal protozoa.

It was a simple matter to show that a cellulase was present in the alimentary tract of *Cryptocercus*. Only a single typical experiment need be detailed. The guts of twenty roaches were ground in a mortar with sand to a brown paste. This was diluted with distilled water to 25 cc. giving a light brown suspension, which, after filtration through fine filter-paper, became almost white. The extract, covered with toluene, was dialysed in a collodion bag against running water for 18 hours to rid it of all reducing substances. Two tubes were then prepared, one containing 1 cc. of the extract, the other 1 cc. of the extract with a small amount of reprecipitated, powdered cellulose. The liquid in both was covered with toluene and the tubes were incubated at 35·5°. Tests were made for glucose with Benedict's reagent.

	Benedict's test after	
Tube contents	4 hours	24 hours
1 cc. extract	–	–
1 cc. extract + cellulose	+	+ + +

– means test negative.

An active extract could also be obtained by grinding up the gut of a single roach, diluting to 5 cc. and centrifuging.

In several cases, the digest, after the action of the enzyme on the cellulose had ceased, was evaporated to dryness over a water-bath, and the residue taken up in phenylhydrazine-sodium acetate solution. On boiling, typical glucosazone crystals were obtained. In one experiment these were filtered off, washed and dried, and the melting-point found to be 202–204° (uncorr.).

It next appeared important to determine whether the cellulase was secreted by the protozoa, or by the host, or by both. Insect enzymes are typically produced in the midgut and in the salivary glands [Abbott, 1926; Wigglesworth, 1927]. The flagellates of *Cryptocercus* are present only in the hind-gut [Cleveland, Hall and Sanders, unpublished]. The following experiment was accordingly performed. The salivary glands, fore- and mid-guts, and hind-guts of 5 roaches were carefully removed, and a separate extract made from each. The salivary glands, after having been ground up, were diluted with water to 3 cc., the fore- and mid-guts to 3 cc., and the hind-guts to 15 cc. The extracts were partly cleared by centrifuging.

	Benedict's test after	
Tube contents	1 day	3 days
1 cc. salivary gland extract, 0·5 cc. 95 % alcohol	–	–
Same with cellulose	–	–
1 cc. fore- and mid-gut extract, 0·5 cc. 95 % alcohol	–	trace
Same with cellulose	–	trace
1 cc. hind-gut extract, 0·5 cc. 95 % alcohol	–	–
Same with cellulose	+ +	+ +

The enzyme is thus present only in the hind-gut, a fact which indicates its protozoan origin.

Still more conclusive proof of the protozoan origin of the enzyme was obtained from the following experiment, done in conjunction with Dr L. R. Cleveland. A group of roaches was fed for 1½ months on nearly pure cellulose (Whatman filter-paper No. 40). The roaches were then divided into three groups. One group (*A*) received no further treatment. A second (*B*) was defaunated by keeping the insects at a temperature of 34° for 6 hours, a procedure which in no way injures the roach. The third group (*C*) was defaunated by oxygenation at 4 atmospheres for 2 hours [Cleveland, 1925]. An extract from a roach of group *A* was oxygenated at the same time with the insects of group *C*. The latter, after treatment, were fed on the alimentary canal material of roaches killed and defaunated by keeping them at a temperature of − 10° for 1 hour. This was done so that the group *C* roaches could be reinfected by any bacteria that might have been killed by the oxygen treatment (bacteria are generally very resistant to low temperatures) and thereby have a normal intestinal flora. Extracts were made from each group, using the gut of one roach diluted to 5 cc. and centrifuged, with results as summarised below.

No.	Tube contents	Benedict's test after 1 day	2 days	3 days
1	1 cc. from *A*, 0·5 cc. 95 % alcohol	−	0	0
2	Same as 1 with 0·25 cc. cellulose suspension	+ +	0	0
3	Same as 1 oxygenated 2 hrs., 4 atm.	−	0	0
4	Same as 2 oxygenated 2 hrs., 4 atm.	+ +	0	0
5	1 cc. from *B*, 1 day after treatment, 0·5 cc. 95 % alcohol	−	−	−
6	Same as 5 with 0·25 cc. cellulose suspension	−	−	−
7	1 cc. from *C*, 2 days after treatment, 0·5 cc. 95 % alcohol	−	−	−
8	Same as 7 with 0·25 cc. cellulose suspension	−	−	−

0 means not tested.

It is evident that regardless of the method of defaunation used, the enzyme disappears coincidently with the disappearance of the protozoa, although the treatments which destroy the protozoa injure neither the enzyme extract nor the roach. The roach itself secretes no cellulase, a fact which is very important with respect to the symbiotic nature of the flagellates.

Still further proof that the cellulase is secreted by the flagellates was furnished by the action of an extract prepared from cultures of *Trichomonas termopsidis*, a xylophagous, intestinal flagellate of the termite, *Termopsis angusticollis*. This organism, now in its 22nd subculture, has been cultivated for almost 2 years in an artificial medium containing finely powdered cellulose[1]. The cultures are contaminated by only one species of bacterium, a small bacillus which actively ferments dextrose but is incapable of attacking cellulose. The contents of 20 culture-tubes were centrifuged, and the small mass of protozoa thus obtained was ground with sand, diluted to 2 cc. and cleared by centrifuging. The results were:

Tube contents	Benedict's test after 2·5 hrs.
1 cc. extract, 0·5 cc. phosphate buffer (p_H 5·3), 0·5 cc. water	−
1 cc. extract, 0·5 cc. phosphate buffer (p_H 5·3), 0·5 cc. cellulose suspension	+ +

[1] Full details of the cultivation will be given in a later paper.

Presence of the cellulase in termites.

The guts of 100 *Reticulitermes flavipes* were ground with sand and diluted with water to 3 cc. The enzyme was adsorbed on aluminium hydroxide and eluted with 3 % KH_2PO_4.

Tube contents	Benedict's test after 5 hours
0·5 cc. extract, 0·5 cc. buffer (p_H 5·3), 0·25 cc. cellulose suspension	+ +
Same but without cellulose	–

The gut of a single *Termopsis angusticollis* was ground with sand and diluted with water to 2 cc. This extract also showed typical enzyme action.

Properties and partial purification of the cellulase.

The properties of this protozoan cellulase have been studied in some detail, mainly to provide a basis for comparison of this enzyme with cellulases from other organisms, notably snail-cellulase and malt-cellulase.

Very early in the course of the work it was noted that the physical state of the cellulose had much to do with the ease with which it was attacked by the enzyme. Thus an extract of 25 *Cryptocercus* guts dialysed 24 hours and made up with alcohol and distilled water to 25 cc. containing 30 % alcohol gave these results (incubated at room temperature):

	Benedict's test after		
Tube contents	1 day	2 days	3 days
5 cc. extract, ground filter-paper	–	–	+ +
5 cc. extract, cellulose suspension	+ +	+ + +	0
5 cc. extract	–	–	–

The extract of a single roach gut diluted to 6 cc. with distilled water gave:

Tube contents	Benedict's test after 1 day at room temperature
1 cc. extract, cellulose suspension	+ + +
1 cc. extract, filter-paper	+
1 cc. extract	–

The material referred to as cellulose suspension was prepared by v. Weimann's method in accordance with the directions given by Pringsheim and Bauer [1928]. 1 g. of pure filter-paper was dispersed in a solution of 50 g. of lithium chloride in 50 cc. of water at a temperature of 160°. The solution so obtained was poured into about 600 cc. of cold water, whereupon the cellulose precipitated out in a very fine flocculent form. It was washed and centrifuged about 9 times with water. The final residue was taken up in distilled water to give a suspension having about 6 to 8 mg. of cellulose per cc. The surface of the cellulose exposed to enzyme action by such a preparation is much greater than that exposed by even finely powdered filter-paper and the micellar structure is probably also different. Karrer, Schubert and Wehrli [1925] have shown that both these factors are important with respect to the degree of hydrolysis of

various cellulose preparations by snail-cellulase. Any form of treated cellulose, although chemically identical with native cellulose, was more easily hydrolysed than the latter.

The enzyme concentration also affected the extent of hydrolysis. A series of tubes was prepared in which a watery extract of the cellulase was diluted 1–4, 1–8, 1–16, *etc.* to 1–128 with phosphate buffer at a p_H of 5·3. The results were:

Dilution of extract	Roach units per cc.	Benedict's test after 2 hours
1–4	5/6	+ + + + +
1–8	5/12	+ + + +
1–16	5/24	+ + +
1–32	5/48	+ +
1–64	5/96	+
1–128	5/192	–
1–4 (no cellulose)	5/6	–

One roach unit is the extract from one roach. This term will be used to give an approximate idea of the concentration of material in the various extracts. It has meaning only when preparations from one and the same extract are being compared. Some quantitative results indicating the effect of concentration of enzyme on the time course of the reaction, cellulose → glucose, are given in a later portion of this paper.

The cellulase was inactivated by 10 minutes' immersion in a boiling water-bath, as well as by 1 hour's at 60°. The extracts were treated first and then were mixed with cellulose suspension and allowed to act on it at a temperature of 35° for periods of time up to 24 hours.

It has been possible partially to purify the enzyme extracts. The original watery preparations are always smooth brown suspensions which pass through coarse filter-paper unchanged. Filtration of such an aqueous extract through a Seitz asbestos filter gives a clear colourless filtrate devoid of enzyme. If, however, enough alcohol is first added to the extract to give a 30 % concentration, and the mixture is kept about a day, subsequent filtration through a Seitz filter gives a clear, slightly yellowish filtrate, which does contain the enzyme, although in not nearly as high a concentration as in the unfiltered material. In watery extracts the cellulase appears not to pass through a collodion membrane, as shown below.

Tube contents	Benedict's test after 2 hours	Benedict's test after 1 day
1 cc. extract, 1 cc. phosphate buffer (p_H 5·3), 0·5 cc. cellulose suspension	+ + +	+ + +
1 cc. extract in a collodion bag immersed in 1 cc. phosphate buffer (p_H 5·3) with 0·5 cc. cellulose suspension	–	–

In this respect this cellulase seems to differ from the lichenase of Karrer, Staub and Joos [1924, 1] which did act on lichenin through a collodion membrane. In the present instance the collodion bag was made from Merck's U.S.P. collodion, allowed to dry 10 minutes, fixed in 95 % alcohol and washed in water. The membrane used by Karrer, Staub and Joos may have been prepared in a different manner.

The enzyme was not adsorbed on Merck's powdered animal charcoal, but all the brown material present in the extract could be removed in this way. The cellulase could be adsorbed completely by aluminium hydroxide and eluted with 3 % KH_2PO_4, thus again behaving like snail-cellulase [Karrer, Staub and Joos, 1924, 1]. It could also be precipitated with acetone and dried with ether. The experimental details follow.

One hundred roach guts were ground with sand and diluted with distilled water to 30 cc. The mixture was centrifuged and the supernatant liquid dialysed against running water for 19 hours. Three 5 cc. portions of the extract were now subjected to three different treatments. Part 1 was diluted with distilled water to make 10 cc. To part 2, 5 cc. of acetone were added, giving a brown precipitate which was filtered off, washed twice with ether and allowed to air-dry. Part 3 was treated with aluminium hydroxide paste and filtered. The clear colourless filtrate may be designated as part 3*a*. The residue was washed thrice with 10 cc. of 3 % KH_2PO_4, the final preparation (3) being a colourless, slightly turbid liquid. Test- and control-tubes were now set up in this manner: each control-tube contained 3 cc. of extract, 2 cc. phosphate buffer (p_H 5·3) and 1 cc. of distilled water; each test-tube held the same, except that 1 cc. of cellulose suspension replaced the 1 cc. of water. In the case of part 2 the dry powder was suspended in 6 cc. of water to give a concentration of 1·3 mg. per cc. The material in the tubes was covered with toluene. The tubes were incubated for about 2 hours at 35·5°, and the glucose concentration in each was then determined by the Willstätter-Schudel [1918] iodimetric method [modified by Kline and Acree, 1930]. The results were:

Part No.	Mg. glucose per cc.
1	4·3
2	0·2
3	0·8
3*a*	0

The method of precipitation and drying involves a considerable loss. Adsorption on aluminium hydroxide and elution with KH_2PO_4 also occasion a loss of enzyme due to the incomplete nature of the elution. The activity of the purified enzyme is, however, sufficiently great for its use in quantitative studies.

Effect of enzyme concentration and of temperature.

Because of the restricted amount of material available, quantitative work with this protozoan cellulase has necessarily been limited. Enough results have been obtained to indicate the type of reaction and the way in which it is affected by changes in enzyme concentration and in temperature. The reaction was followed in all cases by the titration of samples, removed at intervals, by the modified Willstätter-Schudel iodimetric method, using 2 cc. of 0·1 *N* iodine solution, 3 cc. of 0·1 *N* NaOH and a 5 cc. Folin burette for the thiosulphate.

The protozoa from 10 *Cryptocercus* were washed and centrifuged in a balanced salt solution in which they could live for long periods of time. The mass

so obtained was ground with sand, diluted to 10 cc. with distilled water and centrifuged. 5 cc. of the extract were mixed with 25 cc. of buffer (p_H 5·3) and 5 cc. of cellulose suspension. The mixture was kept at 35° and 1 cc. samples were removed. The data are plotted in Fig. 1.

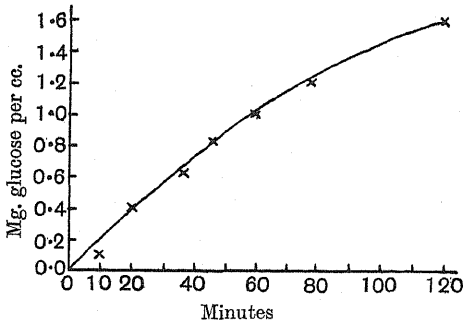


Fig. 1. Hydrolysis of cellulose by an unpurified extract at 35°.

In another experiment the washed protozoa from 30 roaches were similarly extracted. The extract was treated with aluminium hydroxide paste and the enzyme eluted with 30 cc. of 3 % KH_2PO_4. Thus 1 cc. of this preparation contained 1 roach unit. Tubes were set up containing cellulose suspension and enzyme appropriately diluted with phosphate buffer of p_H 5·3. The liquid in each case was covered with toluene and 1 cc. samples were taken. The results are shown graphically in Fig. 2.

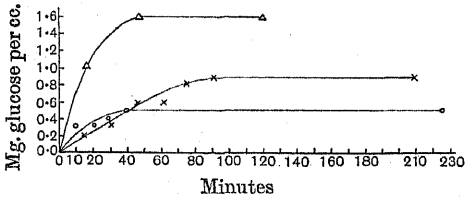


Fig. 2. Effect of enzyme concentration on the hydrolysis of cellulose by a purified extract at 33·5°.

—o— 0·2 roach unit per cc.
—×— 0·5 roach unit per cc.
—△— 0·75 roach unit per cc.

With yet another similarly purified extract, these results were obtained, keeping the concentration of enzyme the same:

Temperature	Mg. glucose per cc. after 2 hours
16°	0·3
21°	0·4
26°	0·7
34°	0·2

A temperature of 26° was thus more favourable, over a period of 2 hours, than any of the lower or higher temperatures tried. For a real analysis of the

temperature effect, it will be necessary to compile a family of curves showing the rates of hydrolysis at various temperatures. The velocity constant of the hydrolysis at each temperature could then be calculated (assuming that the reaction follows the unimolecular law) and the relation of these constants to temperature found.

Cellobiase.

Some cellobiose was prepared from the octa-acetate according to the method of Peterson and Spencer [1927]. It was dissolved in phosphate buffer of p_H 5·3 at a concentration of 0·01 g. per cc., and used in the following experiment.

Washed protozoa from 30 roaches were ground and the mixture centrifuged. The supernatant liquid was made up to 10 cc. with water, giving extract 1, with 3 roach units per cc. 7 cc. of 1 were treated with aluminium hydroxide and the enzyme eluted with 10 cc. of 3 % KH_2PO_4 giving the purified extract 2, with 2 roach units per cc. Two tubes were prepared. One (*A*) contained 2 cc. of 1, 1 cc. of 3 % KH_2PO_4 and 3 cc. of the cellobiose solution to give a mixture having 1 roach unit and 0·005 g. of cellobiose per cc. The other (*B*) held 3 cc. of 2 and 3 cc. of the cellobiose solution giving a mixture with 1 roach unit (purified) and 0·005 g. of cellobiose per cc. The contents of both tubes were covered with toluene. The tubes were incubated at 30·1°. At intervals 1 cc. samples were removed and titrated iodimetrically. The data are summarised in Table I. The figures in column 6 were obtained in the following way. 5 mg.

Table I.

(1) Tube No.	(2) Time (minutes)	(3) Milliequiv. I	(4) Milliequiv. $Na_2S_2O_3$	(5) Total milli-equiv. I used	(6) (see text)	(7) % hydrolysis
A	0	0·1960	0·1466	0·0494	0·0292	0
	15	0·2940	0·2399	0·0541	0·0339	16·2
	30	0·2940	0·2380	0·0560	0·0358	22·8
	45	0·2940	0·2323	0·0617	0·0415	42·7
	135	0·2940	0·2152	0·0788	0·0586	100
B	0	0·2940	0·2599	0·0341	0·0292	0
	15	0·2940	0·2599	0·0341	0·0292	0
	30	0·2940	0·2570	0·0370	0·0321	10
	60	0·2940	0·2513	0·0427	0·0378	30
	120	0·2940	0·2456	0·0484	0·0435	50

of cellobiose react with 0·0292 milliequiv. of iodine. This number was accordingly subtracted from the total milliequiv. used at zero time to give the milliequiv. of iodine used by the mixture itself apart from the cellobiose. This latter figure was then in each case subtracted from the total milliequiv. used to give the numbers in column 6. The reducing power of the mixture itself was presumably due to the presence of glucose and was very much less (almost negligible) in the purified extract than in the unpurified. The percentage hydrolysis was obtained from Table II, constructed after a similar table used by Pringsheim and Bauer [1928]. Fig. 3 illustrates the course of the hydrolysis in tubes *A* and *B*.

About 18 hours after the start of the reaction, the remaining liquid in tube *A* was evaporated to dryness and the residue taken up in phenylhydrazine-

Table II.

% hydrolysis	Mg. cellobiose	Milliequiv. I	Mg. glucose	Milliequiv. I	Total milliequiv.
0	5	0·0292	0	0	0·0292
10	4·5	0·0263	0·525	0·0058	0·0321
20	4	0·0233	1·05	0·0117	0·0350
30	3·5	0·0204	1·575	0·0175	0·0379
40	3	0·0174	2·1	0·0233	0·0407
50	2·5	0·0146	2·625	0·0291	0·0437
60	2	0·0117	3·15	0·0350	0·0467
70	1·5	0·0087	3·675	0·0408	0·0495
80	1	0·0058	4·2	0·0466	0·0524
90	0·5	0·0029	4·725	0·0525	0·0554
100	0	0	5·25	0·0583	0·0583

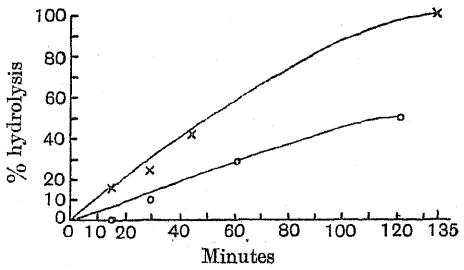


Fig. 3. Hydrolysis of cellobiose at 30·1°.
—×— Unpurified extract (tube *A*).
—o— Same extract purified (tube *B*).

sodium acetate solution. Glucosazone was obtained on boiling and was filtered off while the mixture was hot. The filtrate, on cooling, gave no crystals of cellobiosazone. The remaining liquid in tube *B* was treated in the same manner. In this case both glucosazone and an osazone soluble in hot water (cellobiosazone) were formed.

This protozoan cellulase thus contains, like malt-cellulase and snail-cellulase, a cellobiase.

Summary.

A cellulase has been demonstrated in the symbiotic intestinal flagellates of *Cryptocercus punctulatus*, *Reticulitermes flavipes* and *Termopsis angusticollis*.

The enzyme has also been extracted from one of these flagellates, *Trichomonas termopsidis*, grown for 2 years in pure culture.

The active extract hydrolyses cellulose to glucose. It also hydrolyses cellobiose to glucose. Possibly two enzymes are concerned, a cellulase proper and a cellobiase.

The cellulase complex can be adsorbed on aluminium hydroxide and eluted with 3 % KH_2PO_4. In this and in several other respects it resembles snail-cellulase and malt-cellulase.

Data are presented indicating the way in which the hydrolysis of cellulose by this protozoan cellulase is affected by enzyme concentration and by temperature.

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CCIX. WOOD CELLULOSE.

IV. FURTHER INVESTIGATION OF THE "RESISTANT PORTION" OF THIRIENA PULP.

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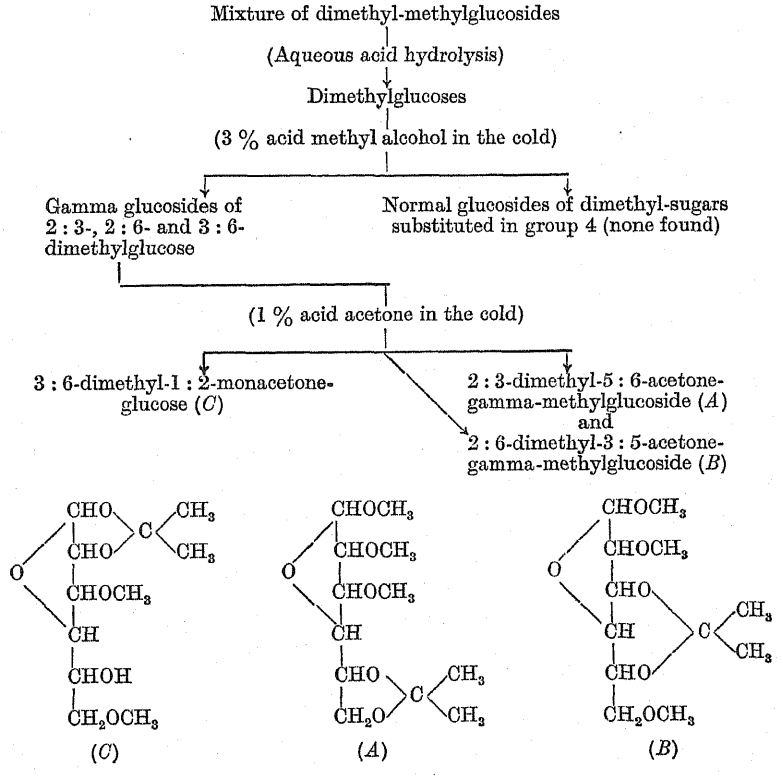
The discovery by the author of the existence within wood celluloses of fractions resistant both to complete etherification and to hydrolysis has already been described in this *Journal* [1932, 1, 2, 3]. The causes underlying this peculiar inactivity of certain of the free hydroxyl groups attached to the glucose residues composing the resistant material could not be explained on grounds either of physical condition or of the positions occupied by these groups. Acetylation of the methylated resistant portions, which had compositions approximating to that of a dimethylcellulose, was easily effected by Barnett's process [1921], when it was found that extensive depolymerisation had accompanied the acylation of the free hydroxyl group. Cleavage of these products, by the action of methyl alcohol containing hydrogen chloride, could now be readily carried out, and it was found that the cleavage products consisted of a mixture of at least five partially methylated glucoses (as the methylglucosides). Individually these substances were 2 : 3 : 6-trimethylglucose; 2 : 3-dimethylglucose; 2 : 6-dimethylglucose; 3 : 6-dimethylglucose; 6-monomethylglucose. Other isomerides of trimethyl- and dimethyl-glucose were absent. No monomethylglucose other than the 6-derivative could be detected, although the absence of isomerides was not proved.

The extremely scanty information at present available regarding the structure of polysaccharides narrowed down to a small range the methods of investigation applicable to the present problem. It was decided to effect a comparison of the dimethyl-sugars produced on hydrolysis of the resistant portions with those derived from the partially methylated non-resistant fractions. To this end a specimen of Thiriena pulp was methylated to a methoxyl content of 27·9 %. Cleavage of the methylate yielded 29·2 % of resistant material, showing a methoxyl content of 19·1 %. Hence, calculation shows that the remaining 70·8 % of non-resistant material had, in the methylate, a methoxyl content of 31·3 %, a value closely similar to the methoxyl content of the fully methylated resistant portion of Thiriena pulp, namely 30·3 %.

The resistant material, after methylation and acetylation, displayed properties identical with those of previous preparations. The mixed glucosides derived from the non-resistant portion contained about 50 % of the dimethyl compounds: after removal of trimethyl derivatives by extraction of an aqueous solution with chloroform, the dimethyl-methylglucosides were purified by the process of extraction from strong potassium carbonate solution [Bell, 1932, 1] and finally distilled in a high vacuum. The resulting colourless syrup was employed in the comparative experiment, the material from the resistant portion having been previously investigated and briefly reported on [Bell, 1932, 3].

The methods employed were those devised by Rutherford [1932], and Oldham and Rutherford [1932], which have been applied to the hydrolysis products of methylated starch by Irvine and Rutherford [1932]. Since complete condensation of the free sugars with methyl alcoholic hydrogen chloride took place in the cold, neither batch of material contained derivatives of glucose with the 4th position substituted. The method of investigation of the mixed glucosides is summarised diagrammatically, as follows.

A. *Estimation of the amount of substitution of the fourth group in the glucose chains. Estimation of the amount of 3 : 6 dimethylglucose.*



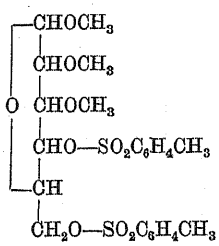
Methoxyl estimation of the mixture indicates the proportion of 3 : 6 dimethylglucose.

B. *Estimation of the amount of 2: 3-dimethylglucose by determination of the amount of free hydroxyl in group 6.*

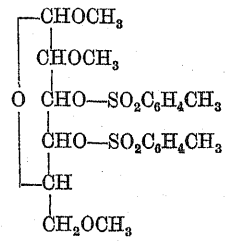
Mixed dimethyl-methylglucosides

↓ (*p*-toluenesulphonyl chloride in pyridine)

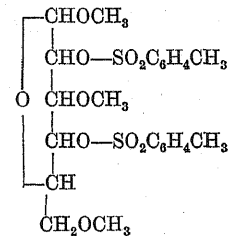
2 : 3-dimethyl-4 : 6-di-*p*-toluenesulphonyl-methylglucoside (*D*)
2 : 6 „ 3 : 4 „ „ (*E*)
3 : 6 „ 2 : 4 „ „ (*F*)



(*D*)

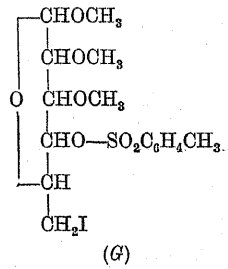


(*E*)



(*F*)

(The above mixture was heated with sodium iodide in acetone whereby the *p*-toluenesulphonyl group in position 6 (*D*) was substituted by iodine with the formation of (*G*). Iodine estimation of the product therefore indicates the amount of 2 : 3-dimethylglucose.)



(*G*)

2 : 3-dimethyl-4-*p*-toluenesulphonyl-6-iodo-methylglucoside

The result of the comparison was striking, for, although the same three individual dimethylglucoses were obtained in both cases, their relative proportions were very different. The three partially methylated glucoses, 2 : 3-dimethylglucose, 2 : 6-dimethylglucose, 3 : 6-dimethylglucose, were in the following relative proportions:

	Portion of the wood cellulose	
	Resistant	Non-resistant
Dimethylglucose	%	%
2 : 3	4·4	20·7
2 : 6	58·6	61·3
3 : 6	37·0	18·0

Experimental.

Tabulated details of the condensation with 1 % acid acetone of the mixtures of the γ-glucosides.

Source of material	Resistant	Non-resistant
Amount of glucosides g.	1·721	1·000
Yield of acetone condensation product g.	1·589	0·862
Yield %	80·0	73·8
Mean OMe (2 determinations) %	31·6	33·6
3 : 6-Dimethylglucose found %	37	18
Theoretical OMe for 3 : 6-dimethyl-1 : 2-monoacetoneglucose		25·0 %
Theoretical OMe for monoacetonedimethyl-methylglucoside		35·5 %

Details of the treatment of the normal glucosides with *p*-toluenesulphonyl chloride are appended. The yields of the products were not good; opportunity did not permit of retreatment of the unreacted materials.

Acylation of the mixtures of dimethyl-methylglucosides.

Source	Resistant	Non-resistant
Amount of starting material g.	2·50	5·47
Yield of di-*p*-toluenesulphonates g.	4·06	8·22
Yield %	68·0	63·2
OMe content %	17·2	17·7
Theoretical OMe for dimethyldi-*p*-toluenesulphonyl-methylglucoside		17·5 %

The estimation of the 2 : 3-dimethylglucose.

Amount of material used g.	3·50	4·46
Yield, after NaI treatment g.	3·09	4·32
2 : 3-Dimethylglucose (by iodine estimation) present %	4·4	20·7
Yield of residue, after $AgNO_3$ treatment g.	3·06 (Theoretical)	3·56 (Theoretical)

Discussion.

As Irvine and Rutherford [1932] found in the case of partially methylated starch, derivatives having the 6th position etherified preponderate in the mixture of dimethylglucoses. In neither case now under consideration were the relative proportions of the different sugar derivatives of the same magnitude as found in starch. Further, although the proportion of 2 : 6-dimethylglucose is the same, whether derived from the resistant or the hydrolysable portions of the wood cellulose, quite marked differences exist between the relative amounts of the 2 : 3- and the 3 : 6-derivatives, indicating that etherification first attacks the 6th group in both cases; in the two fractions of the wood cellulose methylation of groups 2 and 3, together, must pursue different courses when 6 is unsubstituted.

Since 2 : 3 : 6-trimethylglucose is obtained as a cleavage product of the resistant portion, a part of that material must be composed of the ubiquitous polysaccharide unit, the 1 : 5- (or 1 : 4-) anhydroglucose chain. There is at present no evidence that the units giving rise to the dimethylated sugars are not similarly constituted. There must, however, be some peculiar steric effect

produced by the arrangement of the units, since these unetherifiable hydroxyl groups can be easily acetylated. This steric effect would seem, also, to be a property of the fundamental groupings, as the resistant properties persist after depolymerisation of the intact cellulose.

SUMMARY.

A fundamental distinction has been demonstrated between the glucose units composing the resistant and the non-resistant portions of a wood cellulose. Comparison of the three dimethylglucoses (2 : 3-, 3 : 6-, 2 : 6-) derived from the fully methylated resistant portion, and from the non-resistant residue methylated to the same extent as the resistant material, showed that they were formed in different relative proportions, indicating that etherification did not pursue the same course in the two portions of the wood cellulose.

The author is greatly indebted to Sir J. C. Irvine for the interest which he has displayed in this work, and to Mr Walter Caldwell for much practical assistance.

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CCX. CARBOHYDRATE METABOLISM IN HUMAN TRYPANOSOMIASIS.

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During recent years much attention has been directed to the study of blood-sugar changes during trypanosomiasis in animals such as the guinea-pig, rat and rabbit, and it has been considered by many that the changes noted play a significant rôle in the pathogenesis of this disease. The observations of Schern [1925] showed that the life of trypanosomes *in vitro* could be prolonged appreciably by the addition of dextrose or laevulose, and that the serum and the liver of normal animals could reactivate trypanosomes which had almost become motionless, whereas the serum and liver of animals which were in the last stages of trypanosomiasis had much less reactivating power. This author concluded that in trypanosomiasis the blood-sugar and the liver-sugar are gradually used up by the trypanosomes and that a very important pathogenic action of the trypanosomes is a disturbance of the power of the liver to store glycogen, accompanied by a significant hypoglycaemia [*cf.* also review by Schern, 1927]. The same author [1928] found that rabbits and a horse infected with *T. equinum* sometimes showed a marked hypoglycaemia and at other times a hyperglycaemia. Scheff [1928; 1929], as a result of experiments with rats, brought forward similar evidence and concluded that the consumption of sugar and oxygen by the trypanosomes plays a very important rôle in the metabolic disturbance of the host; this disturbance of normal metabolic processes and a state of partial asphyxia are followed, according to Scheff, by an acidosis which contributes to a diminution in the reaction capacity of the host.

Other authors have recorded a hypoglycaemia in rats, mice, guinea-pigs and similar animals infected with trypanosomes, but they are not in complete agreement as to the significance of the phenomenon. Regendanz and Tropp [1927] found that a marked hypoglycaemia exists in rats infected with trypanosomes only in the premortal stages, and conclude that death in these infected rats is not due to a lack of sugar. Regendanz [1929] similarly found that the decreases in the blood-sugar of monkeys infected with *T. rhodesiense* did not run parallel with the number of parasites in the blood. Fenyvessy [1926], Cordier [1927], Knowles and Das Gupta [1928], and Fenyvessy and Scheff [1930], amongst other authors also record a hypoglycaemia in trypanosomiasis of guinea-pigs, rats, *etc.* Dubois and Bouckaert [1927] and Bruynoghe,

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Dubois and Bouckaert [1927] reach the conclusion that the hypoglycaemia is of little or no clinical significance, but Dubois [1928] considers that in certain acute cases of trypanosomiasis in guinea-pigs and mice, death is due to this lowering of the blood-sugar. Zotta and Radacovici [1929], using guinea-pigs infected with *T. brucei*, obtained marked variations in blood-sugar with a constant premortal hypoglycaemia; these authors, however, state that the latter phenomenon is not specific for trypanosomiasis but occurs in a large number of diseases. Linton [1930], working with rats infected with *T. equiperdum*, observed a normal blood-sugar value until a very late period of the disease; the liver-glycogen was found to be markedly decreased. The same author [1929] had previously recorded a normal blood-sugar in rats infected with *T. lewisi*. The relatively enormous sugar consumption by trypanosomes *in vitro* has been well demonstrated by Yorke, Adams and Murgatroyd [1929], who showed that 0·25 cc. of heavily-infected mouse-blood (*T. rhodesiense* infection) when added to 5 cc. of sheep-serum to which glucose had been added caused the disappearance of between 2 and 2·5 mg. of glucose in one hour.

Other authors have investigated the question of excess lactic acid production in trypanosomiasis. Kligler and Geiger [1928] found that in this disease the blood-lactic acid level in the rat shows a progressive rise and that this increase runs parallel with the increase in the number of trypanosomes (*T. evansi*) in the blood. Kligler, Geiger and Comaroff [1929], in a continuation of these investigations, concluded that the increased lactic acid production, as a result of the activities of the trypanosomes, results in an exhaustion of the alkali reserve [*cf.* also Geiger, Kligler and Comaroff, 1930].

These studies of the blood-sugar changes in trypanosomiasis indicate that some disturbance of the normal metabolism of sugar occurs in this disease. Most of the observations have been made, however, on small animals and in many instances with trypanosomes which differ markedly from those pathogenic to man, and it would be unwise to assume, without further evidence, that the results would be applicable to trypanosomiasis in man. Any disturbance of this type in the case of man would, however, be of clinical significance, although it would not necessarily contribute, directly or indirectly, to the decline of the host. No observations appear to have been made so far on the blood-sugar changes in human trypanosomiasis[1], although Sicé [1930] recorded a diminution in the C.S.F.-sugar in nervous trypanosomiasis, with a return, in the majority of cases, to more normal values when treatment was given.

In the present investigation, determinations of the resting blood-sugar values and the sugar tolerance for natives with trypanosomiasis and the values

[1] Since this work was completed and a preliminary report made (Annual Report for 1931 of the Institute for Human Trypanosomiasis Research, Entebbe, Uganda), a review has appeared in the April number for 1932 of the Tropical Diseases Bulletin [**29**, 285] of a paper by Walravens [*Ann. Soc. Belge de Med. Trop.* 1931, **11**, 213]. This author has studied the blood-sugar of 12 sleeping sickness patients before, during and after treatment and has observed no definite variations in the blood-sugar titre during these periods. In two of the patients, the titre was very low before treatment.

for normal natives have been made. The first study and the main part of this investigation was made on sleeping sickness patients kept in the hospital attached to the laboratory, and the controls were natives employed in the laboratory. The latter were selected in such manner that they might be regarded as normal in the sense that they were free from trypanosomiasis; probably every one had had malaria at some time and the majority had been infected with syphilis. The patients were given a liberal diet, the food being selected so that qualitatively and, as far as possible, quantitatively it resembled that of the controls. With the patients, and with practically all the controls, it was possible to be confident that no food had been taken in the morning before samples of blood were taken for fasting blood-sugar determinations. Repeated examinations were made on each patient and the effect of treatment was noted. Twelve patients were thus examined over varying lengths of time, the number available being limited partly by the accommodation but mainly by the disinclination on the part of the native to stay in hospital. With one notable exception, it was extremely difficult to persuade the patients to stay for longer than one month under observation, and as soon as an improvement in their general condition occurred as a result of treatment, they expressed a very strong desire to return to their homes. The results of these laboratory investigations, in spite of the rather small number of patients examined, are considered of value as a more or less systematic study of these selected cases of human trypanosomiasis. The original programme included determinations of the resting blood-sugar of sleeping sickness patients and normals in the West Nile area of the Uganda Protectorate, where it was hoped that a large number of treated and untreated cases might be studied. Owing to circumstances over which the author had no control, however, it was not possible to carry out the second half of this programme.

All the blood-sugar determinations were made by the Hagedorn and Jensen method [1923] [*cf.* Beaumont and Dodds, 1930], this method being selected as the most suitable method for work in the field. A few drops of capillary blood were collected, early in the morning, from a finger of the fasting patient (or control), no food having been taken by the subject since the previous evening. This blood was used immediately for sugar determinations. Duplicate determinations were made with each sample, and the figures given here represent the mean of the two values obtained.

Experimental.

Sleeping sickness patients were selected by the Medical Officers in various parts of Uganda and were sent to Entebbe[1]. These patients were kept in the small hospital attached to the Human Trypanosomiasis Institute at Entebbe,

[1] The author would like to take this opportunity of thanking Major G. J. Keane, Director of Medical and Sanitary Services, the Uganda Protectorate, and Dr S. W. T. Lee for their generous help in this and other matters. In particular, he is indebted to them for the details of the previous history of these patients and for the very satisfactory arrangements made for him by the Medical Department when the author went on safari to the West Nile district in 1931.

and samples of blood were taken from each patient every day (except Sundays) and examined for trypanosomes, malarial parasites, *etc.* Repeated examinations were also made by the red cell adhesion test [Duke and Wallace, 1930; Wallace and Wormall, 1931] and, on occasions, the C.S.F. was collected and examined. In other cases, the blood of the patient was injected into monkeys, guinea-pigs or mice and further examination of the trypanosomes made. The assumption was made that in all these cases the infection was due to *T. gambiense*, since *T. rhodesiense* infections in man appear to be of very infrequent occurrence in Uganda (*cf.*, however, the report of a case of *T. rhodesiense* infection in man in Uganda by Duke [1930]). The results of the red cell adhesion test, the behaviour of the trypanosome in monkeys and other animals (*i.e.* duration of infection and the result of drug treatment) and the symptoms of the patient were, with one possible exception, in entire agreement with the views that the trypanosome concerned was *T. gambiense*. The possible exception was the infection in the patient Kibuka; this matter will be discussed more fully in a subsequent paper [Wormall, 1932].

The patients had a mixed diet which was particularly rich in carbohydrate, the main bulk of the food being taken in the form of sweet potatoes, muhogo flour and sugar cane. Fish was given on most days and meat twice a week. The daily ration included also small quantities of milk, tea and salt. With most of these patients, drug treatment was started after the patient had been at Entebbe for about three or four weeks, the dates of the injections being given in Tables I and II. Treatment during the blood-sugar investigations consisted of the intravenous injection of a solution containing 1 g. of Bayer 205, except in one case where tryparsamide was used. Regular injections of tryparsamide and Bayer 205 were subsequently given to all the patients. At one period during this study, one of the patients developed malaria and was treated with quinine. No blood-sugar determinations were made on this patient during this period or on the seven days following quinine administration.

The patients can be divided into three groups, although it is not suggested that there should be any sharp differentiation between these groups:

(*a*) *Those with little or no previous drug treatment*:
Sumba—no treatment.
Lisuwa, Manika, Oditi and Otenyi (with one injection of tryparsamide each).
Kibuka (with one injection of tryparsamide 2 years previously).

(*b*) *Those with a moderate amount of drug treatment*:
Orachi, Osanda and Atoyo.

(*c*) *Old-standing cases* (*with previous drug treatment*):
Wanjira, Zenesio and Achan.

Age, sex and previous history of these patients.

Sumba. Male, age 25–30 years. Trypanosomes first detected (in the blood) 24. xi. 30. No treatment given prior to arrival at Entebbe, 8. xii. 30.

Lisuwa. Male, age 40. Trypanosomes first detected in Feb. 1931 (gland juice). Received 2·5 g. tryparsamide on 18. ii. 31. Arrived at Entebbe, 28. ii. 31.

Manika. Male, age 20. Trypanosomes first detected in gland juice in Sept. 1930. Received 2·5 g. tryparsamide on 15. ix. 30. Arrived at Entebbe, 27. ix. 30.

Oditi. Male, age 19. Trypanosomes first detected in the gland juice in Feb. 1931. Received 2·5 g. tryparsamide on 18. ii. 31. Arrived at Entebbe, 28. ii. 31.

Otenyi. Male, age 26. Trypanosomes first detected in the gland juice early in Dec. 1930. Received 2·5 g. tryparsamide on 5. xii. 30. Arrived at Entebbe on 21. xii. 30.

Kibuka. Male, age 25–30. Trypanosomiasis diagnosed in 1928, but no further official details were available. (Patient stated that he received one injection only in 1928; none in 1929 or 1930.) Trypanosomes detected in the blood and gland juice on 4. iii. 31. Arrived at Entebbe, 8. iii. 31.

Orachi. Male, age 29. Trypanosomes first detected in the gland juice. Received injections of tryparsamide (2·5 g. each time) on 7. xi. 30, 14. xi. 30, 2. xii. 30 and 9. xii. 30. Arrived at Entebbe, 21. xii. 30.

Osanda. Male, age 10. Trypanosomes first detected in the gland juice 1930. Received 20 injections of tryparsamide (total of 55 g.) from 18. ii. 30 to 15. ix. 30; over practically the whole of this period gland punctures revealed the presence of trypanosomes. This infection appeared, therefore, to be resistant to treatment with tryparsamide, and experiments carried out by Dr J. M. Wallace showed that guinea-pigs infected with this trypanosome were not completely cured by the normal sterilising doses of atoxyl. A large number of adhesion tests were carried out with the serum of this patient and others with the trypanosomes of animals inoculated with his blood, and in all these tests the infection appeared to be a typical *gambiense* infection.

Atoyo. Male, age 8. Trypanosomes first detected in the gland juice in June, 1929. Received 8·5 g. tryparsamide and 1 g. Bayer 205 from 21. i. 31 to 9. iv. 31. Arrived at Entebbe, 22. iv. 31.

Wanjira. Male, age 35. Trypanosomiasis diagnosed in 1926. Received 14 g. tryparsamide and 4 g. Bayer 205 in 1927, 42 g. tryparsamide in 1928, and 6 g. tryparsamide in 1929. In 1930, he received 12 g. tryparsamide during Sept., Oct. and Nov. Sent to Entebbe as a possible case of relapse, 8. xii. 30.

Zenesio. Male. Early history not available. 14 g. tryparsamide and 4 g. Bayer 205 given from May to Sept. 1930. Trypanosomes present in the blood on 12. v. 30 and on several subsequent occasions. Arrived at Entebbe in May, 1930.

Achan. Female, age 40. Trypanosomes detected in gland juice in Aug. 1929. From Aug. to Nov. 1929, she received 35 g. tryparsamide, and from 26. viii. 30 to 9. iv. 31 she received 32 g. tryparsamide and 1·5 g. Bayer 205. Arrived at Entebbe, 22. iv. 31.

Wanjira and Sumba were sent from Tororo (East Province, Uganda); Atoyo and Achan came from Gulu, North Province; all the other patients came from the West Nile district of the Uganda Protectorate.

The results of the fasting blood-sugar determinations with the sleeping sickness patients are given in Tables I and II. The date of each examination and the results of the examination of blood films for trypanosomes are also recorded. Whenever drug treatment (the intravenous injection of a solution of Bayer 205 or tryparsamide) was given, reference is made to it in the Tables in order that a direct study might be made of the influence of this treatment on the fasting blood-sugar level. For convenience the results with patients of group (*a*) are given in Table I and those for groups (*b*) and (*c*) in Table II, but it is not suggested that this division into groups is very clear cut, or that it has any special significance. Table III gives average fasting blood-sugar values for these patients.

In these blood-sugar investigations it was necessary to have, for purposes of comparison, satisfactory values for the fasting blood-sugar of natives of Uganda, and in Table IV the results of such determinations with 12 "normal" natives (four determinations with each native, made at approximately weekly intervals) are given. These control natives were normal in the sense that they did not suffer from trypanosomiasis; all of them were apparently healthy, and

Table I. *Fasting blood-sugar values with trypanosomiasis patients in group (a).*

Sugar values as mg. per 100 cc.

Date	Blood-sugar	Blood examination*	Date	Blood-sugar	Blood examination	Date	Blood-sugar	Blood examination
	Sumba			Lisuwa			Manika	
16. xii. 30	82	+	5. iii. 31	106	+	29. ix. 30	95	–
17. xii. 30	97	+	10. iii. 31	98	+	3. x. 30	98	–
19. xii. 30	82	+	13. iii. 31	102	+	10. x. 30	97	–
22. xii. 30	82	+	18. iii. 31	91	+	13. x. 30	88	–
24. xii. 30	94	+	24. iii. 31	91	+	16. x. 30	99	–
29. xii. 30	89	+	26. iii. 31	98	+	20. x. 30	99	–
3. i. 31	72	+	31. iii. 31	99	+	23. x. 30	1 g. Bayer 205	
9. i. 31	83	+	8. iv. 31	94	–	24. x. 30	100	–
16. i. 31	1 g. Bayer 205		9. iv. 31	1 g. Bayer 205		30. x. 30	88	–
20. i. 31	90	–	15. iv. 31	99	–	31. x. 30	1 g. Bayer 205	
24. i. 31	100	–				1. xi. 30	95	–
29. i. 31	93	–				6. xi. 30	97	–
6. ii. 31	95	–						
	Oditi			Otenyi			Kibuka	
5. iii. 31	105	+	22. xii. 30	69	–	10. iii. 31	93	–
10. iii. 31	96	+	24. xii. 30	86	–	13. iii. 31	100	–
13. iii. 31	100	+	29. xii. 30	93	+	18. iii. 31	90	–
18. iii. 31	87	+	3. i. 31	96	+	20. iii. 31	1 g. Bayer 205	
24. iii. 31	77	–	7. i. 31	1 g. Bayer 205		24. iii. 31	60	–
24. iii. 31	1 g. Bayer 205		9. i. 31	96	–	26. iii. 31	81	–
31. iii. 31	91	–				31. iii. 31	87	–
8. iv. 31	84	–				8. iv. 31	77	–
9. iv. 31	1 g. Bayer 205					9. iv. 31	1 g. Bayer 205	
15. iv. 31	72	–				15. iv. 31	94	–

* Examination of wet and stained blood films for trypanosomes.

Table II. *Fasting blood-sugar values with trypanosomiasis patients of groups (b) and (c).*

Sugar values as mg. per 100 cc.

Date	Blood-sugar	Blood examination	Date	Blood-sugar	Blood examination	Date	Blood-sugar	Blood examination
	Osanda			Wanjira			Zenesio	
6. x. 30	77	+	16. xii. 30	99	–	29. ix. 30	57	–
8. x. 30	91	+	17. xii. 30	105	–	6. x. 30	69	–
10. x. 30	93	+	19. xii. 30	98	–	10. x. 30	70	–
13. x. 30	99	+	22. xii. 30	91	–	11. x. 30	1 g. Bayer 205	
16. x. 30	90	–	24. xii. 30	97	–	13. x. 30	107	–
20. x. 30	93	+	29. xii. 30	100	–	16. x. 30	86	–
24. x. 30	82	–	3. i. 31	96	–	20. x. 30	77	–
25. x. 30	1 g. tryparsamide		9. i. 31	98	–	24. x. 30	79	–
30. x. 30	79	+	20. i. 31	101	–	30. x. 30	73	–
30. x. 30	1 g. Bayer 205		24. i. 31	101	–	1. xi. 30	85	–
1. xi. 30	99	–	29. i. 31	104	–	6. xi. 30	83	–
6. xi. 30	94	–	6. ii. 31	107	–	14. xi. 30	72	–
8. xi. 30	1 g. Bayer 205					25. xi. 30	74	–
14. xi. 30	91	–				27. xi. 30	86	–
21. xi. 30	1 g. Bayer 205							
25. xi. 30	87							
	Orachi			Atoyo			Achan	
22. xii. 30	85	+	29. iv. 31	91	–	6. v. 31	79	–
24. xii. 30	90	+	6. v. 31	86	–	12. v. 31	77	–
29. xii. 30	86	+	12. v. 31	82	–			
3. i. 31	87	–						
7. i. 31	1 g. Bayer 205							
9. i. 31	78	–						

Table III. *Average values for fasting blood-sugar of trypanosomiasis patients.*

Blood-sugar as mg. per 100 cc.

	Before treatment at Entebbe		Before and after treatment (all values)	
Patient	No. of determinations	Average	No. of determinations	Average
Sumba	8	85	12	88
Lisuwa	8	97	9	98
Manika	6	96	10	96
Oditi	5	93	8	89
Otenyi	4	86	5	88
Kibuka	3	94	8	85
Osanda	7	89	12	90
Wanjira	12	100	12	100
Zenesio	3	65	13	78
Orachi	4	87	5	85
Atoyo	3	86	3	86
Achan	2	78	2	78

Table IV. *Fasting blood-sugar (mg. per 100 cc.) normals.*

	Determinations				
	1	2	3	4	Average
Lolenti	86	89	81	84	85
Joseph	107	93	95	98	98
Mikaili Mayanja	83	82	88	90	86
Butulumayo	88	94	85	85	88
Matayo Kiwanuka	92	85	102	99	94
Yusufu Kolokojo	99	94	96	100	97
Abinayo Kitakule	104	100	99	109	103
Yokana	97	101	98	92	97
Alajabu	89	92	93	88	90
Asiri Mutasa	77	95	75	82	82
Erika Kasozi	98	81	85	88	88
Isaka Serwangu	94	104	82	96	94

Table V. *Carbohydrate tolerance tests.*

Blood-sugar values (mg. per 100 cc.) after taking 50 g. glucose.

	Before glucose	½ hr. after glucose	1 hr.	1½ hrs.	2 hrs.	3 hrs.
Manika	98	111	166	141	107	100
Osanda (1)	91	137	131	124	108	91
,, (2)	90	104	100	102	88	77
,, (3)	93	125	121	90	70	91
Zenesio (1)	70	107	117	105	105	77
,, (2)	72	97	148	109	105	74
,, (3)	74	124	162	122	115	87
Sumba (1)	97	102	127	119	112	94
,, (2)	94	104	127	135	130	105
Lisuwa (1)	94	123	152	114	89	87
,, (2)	99	153	166	97	92	93
Normal native (1)	84	109	101	100	88	77
,, (2)	86	161	201	181	115	90
,, (3)	81	123	111	106	74	82
,, (4)	91	125	121	110	89	90
,, (5)	92	131	137	129	92	85

were not suffering from malaria during the period when their blood-sugar values were determined.

In view of the suggestion made by several authors that in trypanosomiasis of small animals there is a general disturbance of carbohydrate metabolism involving a derangement of the storage of glycogen by the liver, it was decided to make glucose tolerance tests with certain of the sleeping sickness patients and normal natives. Table V gives the results of the tests with the patients and a few typical results with controls. None of the patients and only one of the controls (normal native No. 2 in the Table) passed sugar in the urine during these tolerance tests.

Discussion.

The results of determinations of the fasting blood-sugar of natives suffering from trypanosomiasis (Tables I and II) indicate that in this condition the fasting blood-sugar is not markedly lower than that for a native who does not suffer from this disease (*cf.* Table IV for the controls). Marked variations from time to time in the fasting blood-sugar of any one patient certainly occur, but such variations also occur with normal natives. The fluctuations are perhaps somewhat greater with the patients, but the difference between the patients and the controls in this respect does not appear to be significant. Nor are the average values for the sleeping sickness patients very definitely lower than the average values for the control normal natives (*cf.* Tables III and IV), and it is only in isolated instances that there is any suggestion of a marked hypoglycaemia.

It is perhaps difficult to decide what might be regarded as the lower limit for normal fasting blood-sugar values. With the controls used in this work (natives of various tribes in Uganda) the lowest value was 75 mg. per 100 cc. In some investigations carried out on two tribes in Kenya (Special Report Series, No. 155, of the Medical Research Council, Edited by J. B. Orr and J. L. Gilks, 1931), figures of 77 to 136 mg. per 100 cc. are given for the fasting blood-sugar values of normal natives, with perhaps slightly lower values in cases of tropical ulcer (one low value of 65 mg.). In the course of another investigation, Dr F. W. Vint and Dr D. Harvey in Nairobi, using a modification of the Hagedorn and Jensen blood-sugar method, obtained, with natives, fasting blood-sugar values which were usually about 100 mg., or slightly more, per 100 cc. The lowest value in 25 determinations was 75 mg. per 100 cc. (private communication from Dr Harvey in 1931). From these figures for normal natives in Uganda and Kenya, it might be considered, therefore, that the following values for the sleeping sickness patients investigated in the work described here were subnormal; 57, 69, 70, 72 and 73 mg. per 100 cc. with Zenesio, 60 mg. with Kibuka and 69 mg. with Otenyi. These low values, although they may offer some support for the view that there is a hypoglycaemia in human trypanosomiasis, do not occur sufficiently often to suggest a very marked disturbance of carbohydrate metabolism in this disease. The results of the glucose tolerance tests with the patients and a few

of the controls (Table V) also indicate that, in the early stages of trypanosomiasis at least, there is no very serious inability of the liver to deal with glucose. Several of the sugar tolerance curves were of the lag type, and this finding is in agreement with the observations of Bhatia and Coelho [1925], who carried out tests on Indians. The latter authors obtained values between 83 and 246 mg. per 100 cc. for the fasting blood-sugar of these people accustomed to a vegetarian diet.

On theoretical grounds alone it is perhaps rather difficult to conceive of a hypoglycaemia in man due entirely to a heavy sugar consumption by the trypanosomes. With a supply to the body of 300 g. at the minimum and usually considerably more than 400 g. of carbohydrate daily in the form of food, it appears unlikely that the amount of sugar used by the trypanosomes would, in itself, result in any marked diminution in the fasting blood-sugar level or in the amount of glycogen stored in the liver and muscles of the patient. It may be, however, as has been suggested by several authors as a result of experiments on smaller animals, that the consumption of glucose by the trypanosomes leads indirectly (perhaps as a result of the production of certain metabolites) to a disturbance of the mechanism concerned with maintaining a minimum blood-sugar level, and it is perhaps only to be expected that these disturbances would not be manifested until the infection had been established for some time. For this reason it would be of interest to know whether there are any marked changes (diminution in glucose content, *etc.*) in the C.S.F. of patients in the advanced stages of sleeping sickness.

Intimately linked up with this problem of carbohydrate metabolism is the oxygen consumption of the trypanosomes, and it is not impossible that a serious disturbance of normal metabolic processes occurs as a result of the trypanosomes depriving certain organs and tissues of part of their supplies of carbohydrate and oxygen. In the course of this investigation in Uganda it was noticed that the blood taken from the finger of the trypanosomiasis patient was in practically every case considerably darker than samples of blood from the control natives. This difference was best observed when the blood was collected, with the minimum of shaking, in a very small conical test-glass containing a trace of potassium oxalate, or preferably when the blood was immediately sucked up under a layer of liquid paraffin into a capillary pipette containing potassium oxalate or sodium citrate, and observed against a white background. The darker, less oxygenated, bloods could then be picked out at once. Similar differences were observed in tests with samples of blood from monkeys infected with trypanosomes and normal monkeys. This darkness did not appear to be evident only when trypanosomes were present in the blood, since it was observed with these sleeping sickness patients when no trypanosomes could be detected in wet and stained blood films. Thus it does not appear to be due entirely to the using up for metabolic purposes of part of the oxygen of the oxyhaemoglobin by trypanosomes present in the sample of blood tested. If these samples were then shaken with air they became as red as the control

samples similarly shaken, but when they had stood again for a short time there was usually but not invariably more reduction in the blood from the sleeping sickness patients. With the blood of infected monkeys, and to a lesser extent with the human bloods which contained trypanosomes, this later reduction can undoubtedly be attributed to a diminution of the oxygen content as a result of the oxidation of glucose by the trypanosomes [*cf.* Yorke, Adams and Murgatroyd, 1929, p. 510]. In the instances where no significant number of trypanosomes are present in the blood, these observations seem to indicate a higher reducing power of the plasma. It was intended to carry out experiments along these lines, including determinations of the oxygen content and capacity of arterial and venous bloods of the sleeping sickness patients and the reducing power of the serum or plasma, but unfortunately it was not possible to complete these investigations.

Summary.

1. In view of the findings of various authors, that in trypanosomiasis of small animals there is a significant hypoglycaemia, which according to some is partially, if not entirely, responsible for the decline of the host, repeated blood-sugar determinations have been carried out on 12 sleeping sickness patients in Uganda (*T. gambiense* infections).

2. Determinations carried out with these patients and with suitable controls (natives not suffering from trypanosomiasis) over varying periods of time indicate that in man there is no general hypoglycaemia during the earlier stages of this disease. In certain instances, however, a definitely low blood-sugar value was obtained, these low values being significantly lower than the lowest value with a normal native.

3. Treatment of these patients with Bayer 205 gives variable results in relation to the blood-sugar; sometimes there is a fall, sometimes a rise, and in other cases no change in the blood-sugar level. With the patient with the lowest blood-sugar values, the injection of 1 g. Bayer 205 resulted in a speedy return to normal (or perhaps slightly high) values.

4. No relationship could be demonstrated between the blood-sugar level and the presence in or absence from the blood of trypanosomes.

5. Glucose tolerance tests carried out on a few of the patients and some of the controls do not suggest that in trypanosomiasis there is a very marked impairment of the capacity of the liver to deal with glucose.

The author has pleasure in acknowledging the help which he received at various times during the course of this investigation from Dr H. L. Duke, Director of the Human Trypanosomiasis Institute, and from Drs A. R. D. Adams and J. M. Wallace.

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CCXI. A METHOD OF DETERMINATION OF SOME BIOLOGICALLY IMPORTANT ALDEHYDES AND KETONES, WITH SPECIAL REFERENCE TO PYRUVIC ACID AND METHYLGLYOXAL.

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I. INTRODUCTION.

THE importance of various types of unsaturation in molecules in intermediary metabolism is a subject to which attention is being increasingly devoted. During the study of some of the intermediary mechanisms of carbohydrate metabolism in the liver we have been interested in one type of unsaturated linkage, namely that of the carbonyl group. Aldehydes and ketones possess, as is well known, the capacity of forming hydrazones and osazones, which capacity is sometimes used in their identification and estimation. Another property is their power of forming compounds with bisulphites. This property has long been used as a method of estimating acetaldehyde [Ripper, 1900]. That this method can be used for other aldehydes and ketones was shown by Cook [1930] who based on it a method for the estimation of pyruvic acid.

We have extended the method to other aldehydes and ketones with, we feel, some measure of success and can utilise the method, for example, for the determination of a mixture of pyruvic acid with acetaldehyde or methylglyoxal in a single sample. The method shares the disadvantage of unspecificity with the other methods of estimation of carbonyl compounds, there being inherently no specific method of estimation for a mixture of two carbonyl compounds except in so far as the hydrazones or osazones of acids may be separated from those of non-acidic compounds by their solubility in sodium carbonate [Dakin and Dudley, 1913].

Owing to the considerable attention focused on the position of methylglyoxal in intermediary metabolism numerous methods have been proposed for its estimation. As will be shown later the question of the estimation of methylglyoxal in tissues is rather a difficult one.

Wendel [1932] has given a very complete account of the methods that have been used for the estimation of pyruvic acid. In addition to pure pyruvic acid we have devoted some attention to the subject of the polymerides of this acid.

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We feel that our method offers a great advantage in the rapidity with which estimations can be carried out with the minimum of apparatus and the necessity of only one standard solution.

II. Principle and experimental details of estimations of a single substance.

In the presence of excess of sodium bisulphite the aldehydes and ketones studied react at room temperature to form bisulphite compounds. The principle of the method is to remove the excess bisulphite from the solution by adding the required amount of iodine. The bisulphite compounds are then hydrolysed by saturating the solution with sodium bicarbonate, and the liberated bisulphite is titrated with a dilute standard iodine solution. Thus in general 1 cc. of N iodine is equivalent to half the molecular weight of the aldehyde or ketone expressed in mg. The principle is similar to that described by Clausen [1922] and amplified by Friedemann, Cotonio and Shaffer [1927].

The conditions which we have found most suitable are as follows. A 5 cc. aliquot of the solution to be estimated is added to 1·0 cc. of approximately M sodium bisulphite solution. The solution is allowed to stand for at least 15 minutes and then a few drops of starch solution are added. The excess bisulphite is then removed by running in approximately $N/10$ iodine solution (conveniently from a burette) until the blue colour indicates the presence of a slight excess of iodine. The blue colour is discharged by the addition of a few drops of approximately $N/100$ sodium thiosulphate solution. The end-point is then adjusted to a faint blue colour by means of the dilute standard iodine solution ($N/100$, $N/200$ or $N/500$ according to the concentration of the carbonyl compound). 5 cc. of an aqueous suspension of sodium bicarbonate are then added and the standard iodine again run in until the blue colour of the indicator is of the same intensity as it was before the addition of the bicarbonate.

It is important that the standard iodine should be freshly diluted with oxygen-free water and also that the bicarbonate suspension should be free from oxygen. The latter end is readily achieved by blowing a stream of carbon dioxide through it for a few minutes immediately before use.

In most cases the reaction is stoichiometric, but where this is not the case an empirical relationship has been found as described below.

With most of the substances studied the reaction between the carbonyl compound and the sodium bisulphite is complete within a very few minutes. With certain substances (notably acetaldehyde and methylglyoxal) it is necessary to allow the solution to stand for 15 minutes.

It is also important when dealing with solutions of biological origin that the reaction should be slightly acid before adding the bisulphite. (See note in section VIII on the effect of glucose on the titration.) The acidity should not, however, be greater than $N/10$, as low results are obtained in strongly acid solution.

With methylglyoxal the acidity of the 5 cc. aliquot should not be greater than $N/50$. The acid most likely to be present in solutions of biological origin is trichloroacetic acid. Table I shows the effect of various concentrations of this acid on the estimation of methylglyoxal.

Table I.

Mg. methylglyoxal		Normality of trichloroacetic acid	% of theory
Added	Found		
1·16	1·07	0·122	92
1·16	1·12	0·061	97
1·16	1·16	0·024	100
0·436	0·419	0·122	96
0·436	0·436	0·024	100

III. Principle and experimental details of the estimation of a mixture of an alkali-stable and an alkali-labile substance.

While certain of the biologically important carbonyl compounds are both non-volatile and stable towards hot dilute alkali, the majority disappear nearly completely when the solution is heated to 100° with dilute alkali in an unstoppered flask. We have found it possible to make use of this difference in properties to effect an approximate estimation of the components of a mixture of such an alkali-stable compound with one which is destroyed by alkali.

With the alkali-stable compound which we have investigated, namely pyruvic acid, the percentage remaining after treatment with alkali under the conditions described below is 93 %, while with the alkali-labile compounds (methylglyoxal, acetaldehyde, glyceraldehyde, dihydroxyacetone *etc.*) a few per cent. only remain. The method is to titrate an aliquot in the usual manner and to titrate another aliquot after heating with alkali.

It is found that the fraction remaining undestroyed is independent of the initial concentration in the case of the stable compound, and approximately a rectangular hyperbolic function of it in the case of the labile compounds. Thus if y mg. of the labile compound are present, and f is the fraction remaining after treatment with alkali,

$$yf = K.$$

The constant K has been determined for a number of compounds (see section V).

Let F = fraction of the stable substance remaining after treatment with alkali,

x = mg. of the stable substance originally present,

1 cc. $N/10$ I = p mg. alkali-labile substance,

1 cc. = q mg. alkali-stable substance.

Then if the titrations before and after the alkali treatment are T_1 and T_2 cc. respectively,

$$x = \frac{q}{F}\left(T_2 - \frac{K}{p}\right)$$

$$y = p\left(T_1 - \frac{T_2}{F}\right) + \frac{K}{F}.$$

The conditions we have chosen for the treatment with alkali are as follows. A 5 cc. aliquot of the solution is placed in a 125 cc. Erlenmeyer flask together with a drop of thymolphthalein solution. 3 cc. of N NaOH are then added in excess of the amount required just to cause the solution to turn blue. The flask is then placed for 1 hour on the boiling water-bath. At the end of this time the contents of the flask are rendered slightly acid with N H_2SO_4. The exact concentration of alkali and the exact time of heating are not of great importance, at least in the case of methylglyoxal. Table II shows the effect of varying these factors.

Table II.

5 cc. aliquots of methyglyoxal, each containing 0·30 mg.

cc. N NaOH	Time of heating (hours)	Fraction remaining
0·5	1·0	0·045
1·0	1·0	0·030
2·0	1·0	0·027
4·0	1·0	0·030
3·0	0·5	0·033
3·0	1·0	0·034
3·0	1·5	0·034
3·0	2·0	0·033

IV. Examples of the estimation of a single substance.

(1) *Pyruvic acid.*

Pyruvic acid (B.D.H.) was repeatedly distilled *in vacuo* to remove the α-keto-γ-valerolactone-γ-carboxylic acid which may constitute up to 30 % of the average commercial sample of pyruvic acid. The acid was frozen out and the crystals, M.P. 10° (approx.), dissolved in water. No attempt was made to obtain this very deliquescent substance in an anhydrous condition, and the stock solutions were standardised by the method of Wieland [1923]. The results obtained by this method agreed perfectly with those obtained by titration with alkali.

Table III shows the results of bisulphite titrations of the stock solutions.

Table III.

1 cc. *N*/10 I≡4·4 mg. pyruvic acid.

Mg. pyruvic acid		
Used	Found	% yield
22·0	22·0	100
5·58	5·49	98
2·79	2·88	103
2·20	2·20	100
1·39	1·35	97
0·697	0·700	100
0·349	0·351	101
0·221	0·225	102

Note on certain degradation products of pyruvic acid.

(*a*) *α-Keto-γ-valerolactone-γ-carboxylic acid.* As first observed by Wolff [1899] pyruvic acid undergoes spontaneous condensation to this substance. As indicated above commercial samples of pyruvic acid may contain up to 30 % of the lactone. Other decomposition products are also present, as indicated by the odour and colour of old samples of the acid. In order to determine the behaviour of these impurities in the method of estimation we have studied the residue which remains after distillation of pyruvic acid *in vacuo*. This syrupy residue crystallises after standing for a few hours *in vacuo* over quicklime [Wolff, 1901], and consists largely of α-keto-γ-valerolactone-γ-carboxylic acid. A solution of the dry substance takes up iodine in the presence of sodium bicarbonate. The end-point is ill-defined, but the amount of standard iodine taken up corresponds to about half that consumed by an equal weight of pyruvic acid. The titre is not increased by previously allowing the solution to stand with sodium bisulphite.

We have observed that dilute solutions of commercial pyruvic acid give a higher bisulphite titration after long standing than when freshly made up. This is probably due to hydrolysis of the lactone to pyruvic acid. Thus a solution with an initial titre corresponding to 28·0 mg./cc. of pyruvic acid gave after standing for 4 months at about 4° in the presence of toluene a titre corresponding to 33·1 mg./cc. Thus it appears that a convenient way of storing pure pyruvic acid is as the aqueous solution of the free acid.

(*b*) *The so-called* "para*pyruvate.*" The so-called *para*pyruvates are formed when neutral or slightly alkaline aqueous solutions of pyruvates are boiled or allowed to stand for long periods at room temperature. We would like to point out that statements have been made that a substance which, on alkaline hydrolysis, reverts to pyruvic acid is formed when a neutral pyruvate solution is incubated for long periods with bacteria [see Quastel and Wooldridge, 1929, p. 133]. The observations of Wolff [1899] and de Jong [1901; 1902] indicate that the so-called *meta-* and *para*pyruvates are formed under the same conditions without the bacteria.

Barium *para*pyruvate was prepared by allowing a saturated solution of crystalline barium pyruvate to stand for several days at room temperature [de Jong, 1902]. The amorphous white solid which separated was washed thoroughly by repeated shaking with water and air-dried to constant weight. The composition of the product thus obtained is, according to de Jong, $C_6H_6O_6Ba, 4H_2O$. The barium was removed by shaking an accurately weighed amount with a slight excess of dilute sulphuric acid, filtering off the barium sulphate, and making up the filtrate and washings to a known volume with water.

The *para*pyruvic acid thus prepared was found to have a capacity of uniting with bisulphite of approximately one-third that of the equivalent amount of pyruvic acid. On this basis, that 1 cc. $N/10$ I$\equiv$13·2 mg. of *para*pyruvic acid[1], the figures given in Table IV were obtained.

Table IV.

mg. *para*pyruvic acid*	
Used	Found
15·3	16·5
7·6	8·2
3·8	3·8
1·9	1·8
0·95	0·80

* Expressed as pyruvic acid.

When aliquots of the solution were heated with alkali as described in section III a strong yellow colour developed. It was not possible to reach a sharp end-point in a bisulphite titration after this treatment. However, the approximate titrations obtained were uniformly higher than before the alkali-treatment.

(2) *Methylglyoxal.*

Methylglyoxal was prepared by the method of Fischer and Taube [1924], immediately redistilled *in vacuo* and weighed by transferring it to a tared glass-stoppered flask of water as soon as it melted. The solutions thus obtained were perfectly clear. The empirical relationship between the amount of iodine used in a bisulphite titration and the weight of methylglyoxal present was perfectly consistent with different preparations. We have, however, been unable to reproduce quantitatively the results obtained by Kuhn and Heckscher [1926] who describe an empirical method of estimating methylglyoxal by oxidation with iodine in a sodium bicarbonate-carbonate buffer mixture. There seemed to us to be an ambiguity in the phrase "eines Gemisches von 0·2 molarer Na_2CO_3- und 0·2 molarer $NaHCO_3$-Lösung." We therefore carried out estimations on our stock solutions according to their method, using (*a*) a mixture containing 0·2 mols. Na_2CO_3 and 0·2 mols. $NaHCO_3$ in one litre, and (*b*) the above mixture diluted to twice its volume with water.

Estimations carried out on a solution containing 9·875 mg. of methylglyoxal yielded the following results:

Mixture (*a*) 13·20, 13·23, 13·23 mg.
,, (*b*) 12·11, 11·75, 11·78 mg.

When methylglyoxal is titrated according to our method, the iodine titre does not correspond to a stoichiometric union of either one or two molecules of bisulphite with one molecule of methylglyoxal, but to an intermediate

[1] Expressed as pyruvic acid.

value. For quantities between 0·2 and 2·0 mg. of methylglyoxal (in 5 cc.) a linear relationship holds between the titre and the amount of methylglyoxal, 1 cc. $N/10$ I being equivalent to 2·85 mg. of methyglyoxal. Over this range the end-points are perfectly sharp, but with greater concentrations of methylglyoxal this is not the case, and the magnitude of the titre indicates the union of more bisulphite with the methylglyoxal.

The results of some estimations are shown in Table V.

Table V.

1 cc. $N/10$ I≡2·85 mg. methylglyoxal.

Mg. methylglyoxal		
Used	Found	% yield
1·99	1·91	96
1·44	1·45	101
0·996	1·01	101
0·718	0·738	103
0·498	0·491	99
0·249	0·251	101

This relationship being an empirical one, it is important that the conditions which we have described should be strictly observed. The volume of the solution of methylglyoxal is 5·0 cc., and the sodium bisulphite is added as 1·0 cc. of *M* solution. The above relationship does not hold when the concentration of bisulphite differs greatly from that specified.

It is also important that the normality of the methylglyoxal solution with respect to acid should not be greater than $N/50$ (Table I).

(3) *Dihydroxyacetone.*

Dihydroxyacetone was prepared by oxidising glycerol through the agency of *Acetobacter suboxydans*[1]. The dihydroxyacetone was isolated from the culture medium according to the directions of Bertrand [1898]. The dimeric compound was purified by washing with alcohol and then distilled at a pressure of 0·05 mm. [Fischer and Mildbrand, 1924]. The monomeric form thus obtained was washed with anhydrous alcohol-ether mixture (1 : 1) to remove traces of glycerol and dried over phosphorus pentoxide; M.P. 72–80° (uncorr.).

Table VI shows the results of the estimations.

Table VI.

1 cc. $N/10$ I≡4·5 mg. dihydroxyacetone.

Mg. dihydroxyacetone		
Used	Found	% yield
6·00	5·79	97
3·00	2·95	98
1·50	1·47	98
0·751	0·755	100
0·375	0·380	101

[1] We wish to express our thanks to Prof. A. J. Kluyver of Delft for kindly supplying us with a culture of *Acetobacter suboxydans*.

(4) dl-*Glyceraldehyde.*

This substance was prepared from glycerol by the synthesis of Wohl [1898]. The details described by Witzemann [1914] were followed, except for the preparation of β-chloropropionacetal, in which the conditions of Reeves [1927], which give a superior yield, were employed; M.P. 139–40° (uncorr.). The solutions were allowed to stand in the presence of toluene and a trace of sulphuric acid for at least 48 hours before estimation, in order that the compound should pass over to the monomeric form. Table VII shows the results of the bisulphite titrations.

Table VII.

1 cc. $N/10$ I≡4·5 mg. glyceraldehyde.

Mg. glyceraldehyde		
Used	Found	% yield
5·86	5·66	98
5·00	4·89	98
2·93	2·78	95
1·47	1·48	101
0·733	0·726	99
0·365	0·354	97

V. Determination of the constants K and F and examples of the estimation of mixtures of pyruvic acid with methylglyoxal or acetaldehyde.

The determinations of the constant K with quantities of the alkali-labile carbonyl compounds varying between 6·0 and 0·2 mg. in each case are summarised in Table VIII.

Table VIII.

		Mean
Acetaldehyde	0·015, 0·007, 0·006, 0·006, 0·006, 0·006	0·008
Glyceraldehyde	0·13, 0·060, 0·030, 0·020, 0·020	0·05
Dihydroxyacetone	0·17, 0·12, 0·17, 0·15, 0·13	0·15
Methylglyoxal	0·014, 0·019, 0·009, 0·031, 0·010, 0·009, 0·010, 0·011, 0·042	0·014
Acetoacetic acid	0·190, 0·068, 0·048, 0·025, 0·029	0·07

The experimental error in these determinations is large owing to the small titrations after the alkali treatment. This however does not greatly affect the accuracy of the estimation, since f is small compared with F.

The figures for acetoacetic acid are based on the initial concentration determined by the Van Slyke method (see section VII).

The estimations of the percentage of pyruvic acid remaining after heating with alkali are somewhat inconsistent. We have been unable to find any explanation of this inconsistency. Triplicate titrations of samples of the same solution after heating with alkali show considerable variations.

Table IX gives examples of the results obtained.

Table IX.

Mg. pyruvic acid (in 5 cc.)	F		
6·0	0·914	0·905	0·992
2·8	0·945	0·960	0·755
1·4	0·869	0·903	0·949
0·7	0·897	0·955	0·940
0·35	0·906	0·926	0·104

The mean of a very large number of determinations gives $F = 0{\cdot}93$.

The values of F obtained for hydroxymaleic acid are shown in Table X. The initial concentration of hydroxymaleic acid is based on the weight taken and not on the bisulphite titration (see section VII).

Table X.

Mg. hydroxymaleic acid (in 5 cc.)	F
7·93	0·82
3·97	0·87
1·98	0·81
0·99	0·91
0·50	0·93

Table XI shows the results obtained in the estimation of mixtures of pyruvic acid with methylglyoxal and acetaldehyde.

Table XI.

Mg. used			Titrations expressed in terms of cc. $N/10$ I	Mg. found		
Pyruvic acid	Methyl-glyoxal	Acetal-dehyde		Pyruvic acid	Methyl-glyoxal	Acetal-dehyde
1·64	—	0·63	$T_1 = 0{\cdot}661$ $T_2 = 0{\cdot}357$	1·67	—	0·62
1·64	—	1·27	$T_1 = 0{\cdot}911$ $T_2 = 0{\cdot}345$	1·61	—	1·19
1·64	0·96	—	$T_1 = 0{\cdot}712$ $T_2 = 0{\cdot}341$	1·59	1·00	—
1·64	1·91	—	$T_1 = 1{\cdot}039$ $T_2 = 0{\cdot}346$	1·64	1·92	—

VI. Examples of the recovery of aldehydes and ketones added to tissues.

Technique.

10·0 g. quantities of the fresh rat tissue were finely chopped. An aliquot of a standard solution of the aldehyde or ketone under test was added, and after about 30 seconds' stirring, 5 cc. of 50 % trichloroacetic acid were added. The mixture was ground for a few minutes with sand and squeezed through muslin. The residue was extracted three or four more times by grinding with small quantities of water and again squeezing through muslin. The filtrate was where necessary (*viz.* in the methylglyoxal estimation) adjusted to about p_H 2 by the addition of N NaOH, made up to 100 cc. and re-filtered. 5 cc. aliquots

were then estimated in the usual manner. Allowance is made in each case for a tissue blank determined at the same time.

Table XII summarises the results.

Table XII.

Tissue	Substance added	Quantity added (mg.)	Quantity found	% recovery
Muscle	Pyruvic acid	66·7	64·8	97·1
,,	,,	7·22	6·96	96·4
Liver	Methylglyoxal	44·1	38·8	88·0
,,	,,	8·75	5·56	63·6
Muscle	,,	18·4	13·5	73·4
,,	Acetaldehyde	12·4	4·34	35·0
,,	Glyceraldehyde	44·6	44·2	99·1
Liver	,,	44·6	43·7	98·0
,,	Dihydroxyacetone	70·0	69·0	98·6
,,	,,	7·61	7·28	95·6

Tissue blanks.

10·0 g. rat-liver ≡ 0·915, 0·474, 0·332 cc. $N/10$ I
,, muscle ≡ 0·336, 0·457, 0·161 ,,

Considering the expefimental difficulty of carrying out a quantitative extraction of the tissues and at the same time keeping the volume of the extract as small as possible, it may be assumed that the recovery of pyruvic acid and of the trioses is practically quantitative.

There are several indications in the literature that dilute solutions of methylglyoxal react in the cold with substances likely to be present in animal tissues. Thus Neuberg and Kobel [1927] showed that this keto-aldehyde reacted with amino-acids forming "melanin" together with CO_2, NH_3 and acetaldehyde. Müller and Pechmann [1889] and also Pinner [1902; 1905] showed that phenylglyoxal readily condensed with ammonia. The reaction of phenylglyoxal with a constituent or constituents of the pancreas was also studied by Giršavičius [1930].

The failure to effect a quantitative recovery of acetaldehyde from muscle may be connected with the observation of Fabre [1925] that while a deproteinised blood-filtrate yields about 0·77 mg. of acetaldehyde per litre when this substance is distilled from the liquid in a current of nitrogen, the same filtrate only yields 0·48 mg. per litre when allowed to stand in the same closed airspace as the Nessler's reagent used for estimating it.

The tissue blanks observed with rat-muscle may be accounted for, at least in part, by the presence of pyruvic acid and methylglyoxal in the filtrates obtained under these conditions [Case and Cook, 1931]. We have made repeated attempts to isolate and identify the substance or substances responsible for the bisulphite-binding capacity of trichloroacetic acid extracts of ox-liver without success. We are at present only able to report that pyruvic acid is not amongst them.

VII. The behaviour of β-ketonic acids and acetone.

An attempt to apply the method to acetoacetic and oxaloacetic acids has shown that these substances cannot be estimated with any great accuracy. Our observations seem however to be worth recording, since they may be of use in a qualitative or semiquantitative manner. The reason for the inapplicability of the method to these substances is twofold. In the first place the bisulphite compounds appear to be sufficiently unstable in acid solution to render the first end-point ill-defined. Secondly a slow formation of iodoform occurs when the solutions of these substances stand in the presence of iodine and sodium bicarbonate. This renders the second end-point ill-defined. We record in Tables XIII and XIV the results of a series of determinations, taking as the end-points a blue coloration which lasted for about 15 seconds.

Table XIII.

Mg. acetoacetic acid		
Used	Found	% of theory*
7·11	6·16	86·6
3·56	3·09	86·7
1·78	1·54	86·5
0·889	0·778	87·4
0·444	0·397	89·4

* Calculated on the basis of 1 cc. $N/10$ I≡5·1 mg. acetoacetic acid.

Table XIV.

Mg. hydroxymaleic acid		
Used	Found	% of theory*
7·93	6·51	82·2
3·97	3·28	82·6
1·98	1·62	81·8
0·991	0·892	90·4
0·496	0·427	86·1

* Calculated on the basis of 1 cc. $N/10$ I≡6·6 mg. oxaloacetic acid.

The sodium acetoacetate solution was prepared free from acetone and ethyl acetoacetate by the method of Shaffer [1921] and standardised by the method of Van Slyke [1917]. The bisulphite titrations and Van Slyke determinations were both made within an hour of the removal of the solution from aeration flask (aeration time was 20 hours).

The oxaloacetic acid (hydroxymaleic acid) was prepared by the method of Simon [1903] from ethyl oxaloacetate and recrystallised from ethyl acetate by the addition of benzene. The substance softened at 145° and decomposed at 156°. The purity was checked by titration with alkali.

30·38 mg. required 4·58 cc. $N/10$ NaOH (phenolphthalein)
Calculated titre = 4·60 ,, ,,

Only freshly prepared solutions were used.

Acetone cannot be estimated owing to the instability of the bisulphite compound in solution.

VIII. Interfering substances.

Reduced glutathione, proteins, glycogen and urea do not interfere with the titration. After heating with alkali however, reduced glutathione solutions have a bisulphite-binding capacity which is probably due to the presence of pyruvic acid which is known to occur amongst the alkaline decomposition products of cystine [see *e.g.* Friedmann, 1903]. For example, 6·85 mg. of glutathione, after treating with alkali, required 0·0385 cc. *N*/10 I.

Unsaturated substances interfere to a small extent in relatively high concentrations. Thus

50 mg.	of	maleic acid	≡ 0·070 cc. *N*/10 I
50 ,,		fumaric acid	≡ 0·017 ,,
50 ,,		cinnamic acid	≡ 0·026 ,,

Bile has a slight bisulphite-binding capacity; *e.g.* 5 cc. of fresh ox-bile took 0·070 cc. *N*/10 I.

Commercial yeast nucleic acid gives a small titre. Thus 50 mg. took 0·022 cc. *N*/10 I.

The behaviour of glucose is remarkable. Neutral solutions of glucose (Kahlbaum) yield a slight titre. 5 cc. aliquots containing the below-mentioned amounts of glucose took the amounts of iodine indicated.

Mg. glucose	cc. *N*/10 I
460	0·15
50	0·05
10	0·01

The end-points are very ill-defined. After heating with alkali the titre is not sensibly increased. Fructose behaves similarly.

In the presence of ammonium salts or amino-acids, however, the titre of glucose is greatly increased. This is only the case if the solution is approximately neutral prior to the addition of the bisulphite. The presence of an amount of acid or base, small compared with the amount of acid represented by the bisulphite, reduces the titre to a value of the same order as it has in the absence of ammonium salts or amino-acids. For example 10 mg. of glucose in the presence of 5 mg. of ammonium chloride have a titre of the order of 0·7 cc. *N*/10 I. In the presence of *N*/100 HCl or *N*/100 NaOH this titre is reduced to 0·01 cc.

It is hoped to publish an investigation of this remarkable effect in a later communication.

Summary.

1. A method of estimating pyruvic acid and methylglyoxal depending on the capacity of these substances to form compounds with bisulphite is described. The minimum amount of these substances to which the method is sensitive is 0·2 mg. of either substance in 5 cc.

2. From the fact that methylglyoxal and other aldehydes and ketones are destroyed by heating in alkaline solution while pyruvic acid and certain other ketones are not, a method has been devised by means of which the components of a mixture of one of the former with one of the latter may be determined.

3. The method is extended to the determination of glyceraldehyde, dihydroxyacetone and acetaldehyde.

4. The effect of compounds containing an ethylene linkage or a sulphydryl group has been studied. In general these compounds do not affect the estimation.

5. The methods of preparation of the carbonyl compounds studied are indicated and the question of some polymerides of pyruvic acid is discussed.

6. The method for the recovery and estimation of some of the above compounds in the presence of animal tissues is described.

We wish to thank Sir Frederick Hopkins for his kind encouragement during the course of this research. One of us (R. P. C.) is indebted to the Chemical Society for a grant for the purchase of chemicals.

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CCXII. THE 2:4-DINITROPHENYLHYDRAZONES OF SOME KETONIC ACIDS OF BIOLOGICAL IMPORTANCE.

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(*Received October 2nd, 1932.*)

During the course of some experiments on the carbohydrate metabolism of the liver it was found necessary to prepare the derivatives of certain compounds which were thought to be of possible biological importance. Among numerous derivatives prepared were the 2 : 4-dinitrophenylhydrazones of mesoxalic, oxaloacetic and acetoacetic acids. As these compounds do not appear to have been described hitherto in the literature we record below our observations on their preparation, which requires some care, and their properties. We have also devised a quantitative method by which their identity may be verified.

Mesoxalic acid 2:4-dinitrophenylhydrazone.

Mesoxalic acid was prepared from diethylmalonate by the method of Curtiss [1906]. The acid was converted into the sodium salt and the latter purified by repeatedly precipitating it from its aqueous solution with alcohol.

Slightly more than the molecular equivalent of 2 : 4-dinitrophenylhydrazine dissolved in boiling 2*N* HCl (0·5 g. in 30 cc.) was added to an aqueous solution of sodium mesoxalate. The hydrazone was immediately precipitated in hair-like pale yellow needles. Sufficient strong hydrochloric acid was then added to render the solution 2*N* and the mixture allowed to stand overnight. The hydrazone was filtered off, washed with 2*N* HCl, and dried *in vacuo* over sulphuric acid. The yield was practically quantitative. The substance is appreciably soluble in water (1 part dissolves in approximately 400 of water at room temperature), but is almost insoluble in 2*N* HCl. It may be conveniently recrystallised by dissolving it in water and adding strong hydrochloric acid. The melting-point is 202° (uncorr.) unaltered by further recrystallisation. (Found N 18·73 %; Calculated N 18·79 %[3].)

The precipitation of the hydrazone occurs even in very dilute solution. Thus a precipitate is formed when 17 mg. of 2 : 4-dinitrophenylhydrazine

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[3] This and subsequent analyses by Mr A. Colwel.

dissolved in 1 cc. of 2*N* HCl are added to 4 cc. of a solution containing only 1·5 mg. of mesoxalic acid.

The hydrazone dissolves in saturated sodium carbonate to give a light brown solution from which it can be reprecipitated by acidification with hydrochloric acid. It is readily soluble in ethyl acetate and is completely thrown out from its solution in this solvent by the addition of a small amount of light petroleum. It is slightly soluble in alcohol, almost insoluble in ether. Although it is a malonic acid derivative its solution can be safely heated to 100°. An aqueous solution of the substance, saturated at the boiling-point, was boiled under reflux for 1 hour. On acidifying with hydrochloric acid the hydrazone was recovered unchanged in melting-point. The melting-point of glyoxylic acid 2 : 4-dinitrophenylhydrazone, the formation of which might be expected under these conditions is given by Brady [1931] as 190°.

The substance differs from other 2 : 4-dinitrophenylhydrazones in giving no colour with alcoholic potassium hydroxide.

Acetoacetic acid 2:4-dinitrophenylhydrazone.

Acetoacetic acid was prepared as the sodium salt in aqueous solution and freed from ethyl acetoacetate and acetone by the method of Shaffer [1921]. The hydrazone was prepared as described above, except that the hydrazine was added as a supersaturated solution in 2*N* HCl at room temperature. The yield of crude product was 85 % of the theoretical figure based on an estimation of the strength of the acetoacetic acid solution by the method of Van Slyke [1917]. The substance was purified by dissolving it in saturated sodium carbonate solution (20 cc. per g. of material), filtering from a trace of insoluble material, and precipitating it by the addition of hydrochloric acid. The precipitate was dried *in vacuo* over sulphuric acid. Crystallisation was effected by dissolving it in the minimum quantity of ethyl acetate at room temperature, and adding ten times the volume of ligroin. The substance crystallises in parallel growths of brownish-yellow pointed needles. It melts somewhat indefinitely at 125° (uncorr.) with evolution of carbon dioxide and the formation of a red liquid. The melting-point was unchanged by further recrystallisation. (Found, N 19·62 %; Calculated, N 19·86 %.)

The hydrazone gives a dark brown solution in saturated sodium carbonate and can be precipitated therefrom by acidification. It is slightly soluble in alcohol but almost insoluble in ether. It is practically insoluble in water. The substance gives a red colour with alcoholic potassium hydroxide.

At no stage in its purification should it be heated, either alone or in solution. In solution in ethyl acetate or glacial acetic acid it undergoes decomposition when warmed, considerably below the boiling-points of these solvents, yielding carbon dioxide and acetone 2 : 4-dinitrophenylhydrazone. The latter was obtained in a state of purity simply by two crystallisations of acetoacetic acid 2 : 4-dinitrophenylhydrazone from boiling ethyl acetate. The derivative melted sharply at 126° (uncorr.). The melting-point of the acetone

hydrazone prepared from acetone and once recrystallised from ethyl acetate was 126°. A mixed melting-point of the two specimens showed no change from this figure. The crystals of both specimens were short rhombic prisms. (Found, N 23·71 and 23·81 %; Calculated, N 23·53 %.)

This hydrazone gives a red colour with alcoholic potassium hydroxide.

Oxaloacetic acid 2:4-dinitrophenylhydrazone.

The oxaloacetic acid used was the maleinoid form (melting-point 150° approx.) prepared by the method of Simon [1903]. The conditions described above for the preparation of the acetoacetic acid derivative were employed. The hydrazone separates slowly in glistening rhombic plates. The substance is thus obtained in a pure state, as judged by constancy of melting-point after recrystallisation, by the nitrogen content, and by the alkali titration figure (see below). (Found, N 17·91 %; Calculated, N 17·94 %.)

The hydrazone dissolves in saturated sodium carbonate to give a brown solution from which it can be reprecipitated by acidification. It is readily soluble in ethyl acetate and can be caused to crystallise from this solvent by the addition of ligroin or benzene. It is slightly soluble in alcohol but almost insoluble in ether. One part of the substance dissolves in about 140 parts of water at room temperature. It can be caused to crystallise from its saturated aqueous solution by adding enough strong hydrochloric acid to make the solution 2*N*.

It softens at 205° and melts at 211° with evolution of gas. Like the analogous acetoacetic acid derivative it decomposes when heated, although rather less readily. Thus drying the substance at 110° for 2 hours caused the melting-point to fall to 208°. If a saturated solution of the substance is made in boiling ethyl acetate, crystallisation does not occur on cooling. The addition of light petroleum however causes the separation of a yellow semicrystalline precipitate. Titration with alkali indicates that partial decomposition into pyruvic acid 2 : 4-dinitrophenylhydrazone has occurred. 4·26 mg. of the product required 2·47 cc. of *N*/100 NaOH. The calculated titres for oxaloacetic acid hydrazone and pyruvic acid hydrazone are 2·64 and 1·63 cc. respectively.

Characterisation of small quantities of the 2:4-dinitrophenylhydrazones of ketonic acids by acidimetry.

In spite of the intense colour of these substances it is possible to dissolve them in excess of standard alkali and titrate the solution with standard acid, provided that an indicator is chosen the colour change of which is not masked by the colour change due to the derivatives themselves. Notwithstanding its low p_K, we have found that bromothymol blue gives satisfactory end-points. This titration may be performed with small quantities of material (2 to 5 mg.) weighed on a micro-balance. In the identification of an unknown substance it affords a useful check on the nitrogen determination. Determinations of C and H are not of great value with these derivatives owing to the

fact that different compounds often have very nearly the same percentage composition with respect to these elements.

Examples of these titrations are shown in the table below. In each case the quantity of the substance indicated was weighed out, dissolved in 5 cc. of $N/100$ NaOH, and titrated with $N/100$ acid.

Microtitrations of 2 : 4 dinitrophenylhydrazones.

Parent acid	Weight taken (mg.)	cc. of $N/100$ NaOH	
		Found	Calculated
Pyruvic	3·74	1·42	1·39
Acetoacetic	4·02	1·44	1·42
Oxaloacetic	3·06	1·97	1·96
Mesoxalic	3·07	1·96	1·96

SUMMARY.

1. The preparation and properties of the 2 : 4-dinitrophenylhydrazones of mesoxalic, acetoacetic and oxaloacetic acids are described.

2. A method is given by which these derivatives may be titrated as acids and which may be used in their identification.

We wish to thank Sir Frederick Hopkins for his kind encouragement. One of us (R. P. C.) is indebted to the Chemical Society for a grant for the purchase of chemicals.

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CCXIII. TRIOSE DEHYDROGENASE. I.

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(*Received September 27th, 1932.*)

The existence of the trioses as intermediary compounds in mammalian carbohydrate metabolism has often been postulated. Since *dl*-glyceraldehyde was first synthesised by Wohl [1898] a number of investigations have been made on the changes which it may undergo in the animal body. References to these are given by Stöhr [1932]. It appears that the compound is capable of complete oxidation in the animal body, and that the liver has the power of converting it into lactic acid, glycogen or hexose. The production of *d*-sorbose from *dl*-glyceraldehyde [Embden, Schmitz and Wittenberg, 1913] is readily explicable on stereochemical grounds. This observation is of importance as it appears to indicate that glyceraldehyde may first be converted into dihydroxyacetone under the conditions prevailing in the liver cells.

An even more extensive series of investigations has been carried out on the behaviour of dihydroxyacetone in mammalian metabolism. The liver can convert dihydroxyacetone into glucose or glycogen. This triose, in contradistinction to glyceraldehyde, has the power of relieving insulin convulsions [Reeves and Hewitt, 1926]. It is not however antiketogenic.

Most of the work hitherto carried out on the trioses has demonstrated only their capacity to play a part in the synthesis of hexoses or glycogen, or to undergo internal oxidation-reduction to lactic acid. The possibility that the trioses might arise during carbohydrate catabolism and undergo direct oxidation has not been seriously entertained. The fact that dihydroxyacetone is autoxidisable in aqueous solution has long been known, and Wind [1925] has studied the influence of heavy metal ions on the autoxidation of both trioses in alkaline phosphate buffer. Euler, Eriksson and Brunius [1928] showed that dihydroxyacetone solutions were capable of reducing methylene blue in the presence of phosphate buffer. As far as we are aware the only observation hitherto made on the catalysis of triose dehydrogenations by an enzyme of mammalian origin is that of Lebedev [1926] who showed that the purified Schardinger enzyme preparation from milk catalysed this reaction.

We have studied the catalytic action of a liver extract prepared by the method of Harrison [1931] on the trioses, on acetaldehyde and on hypoxanthine in the presence of methylene blue.

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Experimental.

The reduction velocity as a function of substrate concentration.

In order to establish the identity or non-identity of the enzymes responsible for the dehydrogenations studied by the "competition" method, it was necessary to determine the optimum concentration of each substrate. In the following experiments an enzyme preparation which had been dialysed at 0° nearly free from ammonium sulphate was used. In general 4 to 6 2-litre changes of distilled water were used in the course of 48 to 72 hours. At the end of this time the last dialysate gave only an opalescence with barium chloride solution. 10 g. of the enzyme powder yielded about 40 cc. of solution. Since the enzyme solution was deeply pigmented, the time for apparently complete reduction of the methylene blue was observed. The velocities recorded in the following curves and tables are the reciprocals of the reduction times in minutes multiplied by 100. In every case the contents of the Thunberg tubes consisted of 1 cc. of the enzyme solution and 0·5 cc. of a solution of 1 part of methylene blue in 5000 parts of phosphate buffer. The phosphate buffer was Clark and Lubs p_H 7·2 buffer made up to be 10 times as concentrated as that given by Clark *viz.* 0·5 *M* with respect to phosphate. When diluted with 1 volume of water and 2 volumes of enzyme solution the resulting mixture had a p_H of 7·1 determined electrometrically. The total volume of the contents of the Thunberg tube was made up to 2·0 cc. with the substrate solution and water where necessary. The reduction times were in every case measured at 37°. The reduction time for the enzyme solution alone under these conditions is of the order of 5 hours.

dl-*Glyceraldehyde.* Crystalline glyceraldehyde prepared as indicated in a previous paper [Clift and Cook, 1932] was used in these experiments. Owing to the sparing solubility of the dimeric form in water, the strong solutions

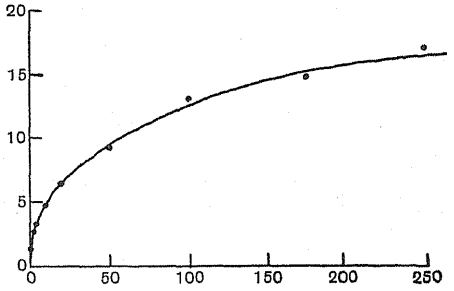


Fig. 1. Initial reaction velocity as a function of glyceraldehyde concentration. Ordinates: relative velocity. Abscissae: glyceraldehyde concentration in millimols. per litre.

necessary were made by dissolving the glyceraldehyde in the minimum amount of water and allowing it to stand at room temperature for several days in the presence of toluene (to prevent infection) until the water could be removed *in vacuo* over sulphuric acid without the glyceraldehyde crystallising out. Although

glyceraldehyde reduces methylene blue in the presence of phosphate buffer, and in the absence of enzyme, control experiments showed that the velocity under these conditions was negligible compared with that of the enzymic reduction. The Michaelis curve for *dl*-glyceraldehyde is shown in Fig. 1. Within the limits of experimental error this curve is represented by the well-known equation

$$v = \frac{Vx}{K_m + x},$$

where v = initial velocity of the reaction,
x = concentration of substrate,
V = velocity of reaction when x is large,
K = Michaelis constant of the system.

Dihydroxyacetone. Freshly prepared solutions of monomeric dihydroxyacetone (prepared as described by Clift and Cook [1932]) were used in every case. Under the conditions described above the velocity of methylene blue reduction by dihydroxyacetone in the absence of the enzyme is not negligible compared to that of the enzymic reaction. Fig. 2 shows the Michaelis curve for methylene blue reduction in the presence of the enzyme. The lower curve shows the relationship between the non-enzymic reduction velocity and the concentration of dihydroxyacetone. Even when allowance is made for the non-enzymic reduction velocity, the curve does not fit the Michaelis equation, the velocity in high substrate concentrations being higher than this equation indicates.

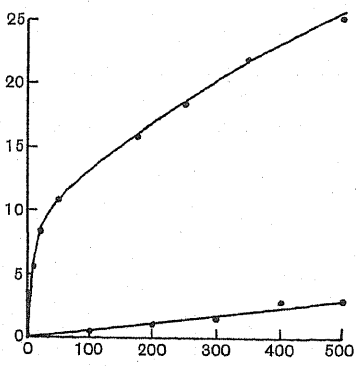


Fig. 2.

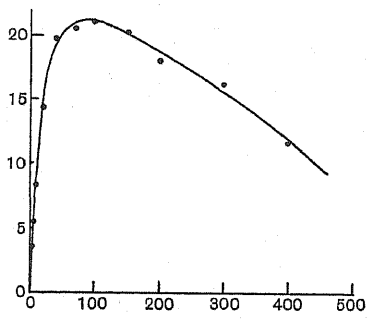


Fig. 3.

Fig. 2. Initial reaction velocity as a function of dihydroxyacetone concentration. Ordinates: relative velocity. Abscissae: dihydroxyacetone concentration in millimols. per litre. Upper curve: in the presence of the enzyme. Lower curve: in the absence of the enzyme.

Fig. 3. Initial reaction velocity as a function of acetaldehyde concentration. Ordinates: relative velocity. Abscissae: acetaldehyde concentration in millimols. per litre.

Acetaldehyde. The relationship of the reduction velocity to the concentration of acetaldehyde is illustrated in Fig. 3. This curve shows that the aldehyde dehydrogenase is inhibited by high substrate concentrations. Acetaldehyde, added in high concentrations to the enzyme preparation causes the appearance

of a precipitate. Since a certain amount of acetaldehyde boils off when the Thunberg tubes are evacuated, no quantitative conclusions can be drawn from the shape of the curve in high concentrations of acetaldehyde. It will be observed that the aldehyde dehydrogenase present in the liver extract has a very different velocity-substrate concentration curve from the Schardinger enzyme studied by Dixon. The optimum aldehyde concentration is 0·1 *M* and the concentration giving half the maximum reduction velocity is 0·013 *M*.

Hypoxanthine. The reduction velocities with various concentrations of hypoxanthine are shown in Table I.

Table I.

Concentration (millimols. per litre) of hypoxanthine	Reduction velocity
1·7	8·3
1·4	8·9
1·0	11·0
0·68	10·9
0·34	10·9
0·17	11·1

The identity of the dehydrogenases.

An attempt to ascertain whether the same or different enzymes are responsible for the dehydrogenations of glucose, the trioses, acetaldehyde and hypoxanthine has been made by allowing the preparation to act on these substrates taken in pairs each at the optimum or nearly optimum concentrations. According to the generally accepted theory of enzyme action, if the same enzyme is responsible for the oxidation of two substrates the reduction velocity for a mixture of the two substrates in optimum concentration should lie between the reduction velocities for the two substrates separately. If different enzymes are responsible, then the velocity for a mixture should be the sum of the velocities for the two components separately. In the results of such experiments shown in Table II, the above conditions were used. The concentrations of the various substrates were: glucose 0·25 *M* [Harrison, 1931], dihydroxyacetone 0·2 *M*, glyceraldehyde 0·15 *M*, acetaldehyde 0·1 *M*, hypoxanthine 0·0007 *M*. Since the velocity-substrate concentration curves for the trioses show that the reaction velocity continually increases with increasing concentration of the substrate, it is to be anticipated that if the same enzyme is responsible for both dehydrogenations the velocity for the dehydrogenation of the mixture of the ketose and the aldose will be somewhat greater than the mean of the velocities for each separately. Table II shows this to be the case. Hence it may be tentatively concluded that the same enzyme is responsible for catalysing both reactions.

The reduction velocity for a mixture of glucose with either triose is slightly less than the sum of the velocities for the components separately. This discrepancy is probably not due to experimental error since a number of experiments gave figures similar to those quoted. Since, however, the velocity for the mixture is considerably greater than the velocity for either glucose alone

Table II.

Substrate	Reduction time mins.	Observed velocity	Sum of observed velocities
Glucose	11·9	8·4	
Glyceraldehyde	8·1	12·4	
Glucose + glyceraldehyde	5·9	17·0	20·8
Glucose	14·3	7·0	
Dihydroxyacetone	7·6	13·2	
Glucose + dihydroxyacetone	5·5	18·2	20·2
Glucose	9·8	10·2	
Acetaldehyde	3·2	31·2	
Glucose + acetaldehyde	3·2	31·2	41·4
Glucose	11·1	9·0	
Hypoxanthine	5·6	17·9	
Glucose + hypoxanthine	3·9	25·6	26·9
Glyceraldehyde	7·4	13·5	
Dihydroxyacetone	6·3	15·9	
Glyceraldehyde + dihydroxyacetone	4·9	20·4	29·4
Glyceraldehyde	7·4	13·5	
Acetaldehyde	3·0	33·3	
Glyceraldehyde + acetaldehyde	3·1	32·2	46·8
Glyceraldehyde	7·4	13·5	
Hypoxanthine	3·8	26·4	
Glyceraldehyde + hypoxanthine	2·6	38·5	39·9
Dihydroxyacetone	7·7	13·0	
Acetaldehyde	4·0	25·0	
Dihydroxyacetone + acetaldehyde	4·5	22·2	38·0
Dihydroxyacetone	6·2	16·1	
Hypoxanthine	3·8	26·3	
Dihydroxyacetone + hypoxanthine	2·3	43·5	42·4
Acetaldehyde	3·6	27·8	
Hypoxanthine	5·6	17·9	
Acetaldehyde + hypoxanthine	4·4	22·7	45·7

or a triose alone, it seems probable that different enzymes are responsible for the two catalyses. This conclusion is supported by the fact that the activity of the extract on glucose decreases by about 50 % in the course of 8 hours at room temperature, while no difference can be detected in the activity of the extract on the trioses under these conditions.

Methylene blue reductions carried out with mixtures of glucose or a triose with acetaldehyde show that the velocity with the mixture is the same as the velocity with acetaldehyde alone. This seems to indicate that acetaldehyde inhibits the glucose and the triose dehydrogenase. If this interpretation is not put upon the phenomenon, the alternative assumption that the enzymes responsible are identical, leads to contradictory results. Thus with the latter assumption one would argue that the aldehyde dehydrogenase and the dihydroxyacetone dehydrogenase are identical. Since hypoxanthine and acetaldehyde appear to compete for the same enzyme, the hypoxanthine dehydrogenase would be identical with the dihydroxyacetone dehydrogenase. But the competition experiment shows that this is not the case.

Corroborative evidence as to the non-identity of the aldehyde and triose dehydrogenases was obtained by a study of the oxygen uptakes in the Barcroft

differential manometer. Hypoxanthine and acetaldehyde show an oxygen uptake in the presence of the enzyme. The action of the enzyme preparation on the trioses, however, is merely to effect a partial inhibition of the autoxidation which these substances show in phosphate buffer. Harrison showed that the glucose dehydrogenase is anaerobic. It seems possible but unlikely that the same enzyme should be capable of activating certain hydrogen donators with respect to oxygen as the acceptor, while it is unable to do so with others. Thus the competition experiments indicate that the extract contains three distinct dehydrogenases—one acting on glucose, one on the trioses, the third on hypoxanthine and acetaldehyde.

Discussion.

The work of Neuberg and his school has shown that methylglyoxal is a probable intermediary between polysaccharides and lactic acid in mammalian metabolism. A probable main course of the oxidative degradation of carbohydrate in muscle is given by Toenniessen and Brinkmann [1930]. Very little is known at present of the mechanism of carbohydrate oxidation in other tissues. We regard the observation that an enzyme capable of activating the trioses as hydrogen donators exists in the liver as of importance in so far as it indicates that these substances may be involved in the oxidative breakdown of carbohydrate in that organ. This possibility is of course open to the objection that the appearance of these very reactive substances in liver metabolism has never been observed.

Our observation raises the interesting question as to what are the oxidation products of the two trioses when dehydrogenated by the enzyme. The similarity in the behaviour of the enzyme with respect to the two substrates indicates that the product may be the same in both cases. The enzyme possibly acts on one triose only or on a common enolic form. The fact that the relationship between the initial reaction velocity and the dihydroxyacetone concentration approximates to linearity in high substrate concentrations indicates that under these conditions the factor limiting the velocity is a non-enzymic reaction, thus lending support to this hypothesis. Lebedev [1926] showed that the oxidation product of glyceraldehyde by the Schardinger enzyme preparation was probably glyceric acid. Our observations agree with his in so far as the enzyme acts more rapidly on the aldose than on the ketose. Lebedev showed that his enzyme was incapable of bringing about a Cannizzaro transformation of glyceraldehyde into glycerol and glyceric acid. Work is now in progress with a view to deciding whether the liver enzyme is capable of bringing about this change and to identifying the products of the oxidation of the trioses by methylene blue. Preliminary experiments have shown that the oxidation is in both cases attended with the production of more acid than can be accounted for by the HCl liberated by the methylene blue which is reduced.

SUMMARY.

1. An extract made from ox-liver contains an enzyme capable of activating glyceraldehyde and dihydroxyacetone as hydrogen donators to methylene blue.

2. The question of the specificity of the enzymes present in the liver extract is discussed. It is concluded that in the liver extract studied there are three distinct dehydrogenases, one acting on glucose, another on the two trioses and the third on hypoxanthine and acetaldehyde.

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CCXIV. THE OSMOTIC PRESSURE OF GLOBIN.

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The present communication records a series of measurements of the osmotic pressure of globin, the protein constituent of the haemoglobin molecule. A number of derivatives of haemoglobin have been investigated by osmometric methods, and it appears that in the cases of reduced and oxygenated haemoglobin, methaemoglobin [Adair, 1924; 1928] and carbon monoxide haemoglobin, which has been studied also by centrifugal methods [Svedberg and Fåhreus, 1926; Svedberg and Nichols, 1927] the molecular weight is a constant, equal to about 67,000.

The preparation of denatured globin from haemoglobin was described by Schulz [1898]. More recently, several methods of obtaining native globin have been suggested [Hill and Holden, 1926; Hamsik, 1930; Anson and Mirsky, 1930 and others]. Most of these involve the treatment of haemoglobin with acids and some organic solvent, whereby the pigment haematin is removed. Roche [1930] has found that at least three different globins are formed when haemoglobin is treated with acids, namely, native globin, fully denatured globin and an intermediate substance, insoluble at the isoelectric point, which has been termed paraglobin. Some measurements of the osmotic pressures of paraglobin solutions are also recorded in this paper.

I. Methods.

In this work, globin has been prepared by the methods of Hamsik [1930] and of Anson and Mirsky [1930]. A preparation of native globin (No. 6) was made by a slight modification of Hamsik's method, as follows. The haemoglobin of the ox, prepared by the ether and salt method of Adair [1925], was precipitated by the addition of acetone, crystals not being obtained. The precipitate was then treated with acetone containing oxalic acid, washed on a Büchner funnel with pure acetone and dissolved in water. The solution obtained was neutralised with 1 % ammonia, whereupon the paraglobin was precipitated, and the filtrate, containing native globin, was dialysed against distilled water. The preparation was carried out at 0° and yielded a slightly pigmented solution of globin.

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Other preparations were made by the methods described by Anson and Mirsky. 0·1 *N* hydrochloric acid was added to an equal volume of 10 % ox-haemoglobin, both solutions being ice-cold, and the globin was precipitated by acetone containing 1 % of *N* hydrochloric acid. After washing the precipitate with pure acetone and then removing as much acetone as possible by suction and pressing between filter-papers, the globin was dissolved in water without previous drying in air, and, in the case of preparations 7 and 9, the solutions were slowly neutralised with 0·1 *N* sodium hydroxide, whereby most of the paraglobin was precipitated; this was finally removed by subsequent addition of 0·4 % ammonium sulphate to the filtrate. The preparation of globin from crystallised and uncrystallised horse-haemoglobin is described in Section V.

In the case of preparations of ox-globin Nos. 1, 2, 3 and 5, and of sheep-globin, the solution of protein, obtained after dissolving the acetone precipitate in water, was dialysed against an acetate buffer of p_H 5·6. As will be described later, the majority of such protein solutions appeared to consist of paraglobin at the end of the period of dialysis. Additional preparations of paraglobin were made by redissolving in a minimum quantity of acid the precipitate formed on neutralisation of solutions containing mixtures of globin and paraglobin.

In the case of ox-globin, we were able to obtain by the method of Anson and Mirsky protein solutions in which the presence of haematin was undetectable. Attempts to prepare sheep-globin by this method yielded in general appreciably pigmented solutions. In our hands, the method of Anson and Mirsky gave large yields of native globin, but we found the native protein to be relatively unstable, even when kept rigorously at 0°.

Osmotic pressure measurements were obtained by the method of Adair [1925], the protein solutions in rigid collodion membranes being equilibrated with standard buffer solutions at 0°. Series of experiments were carried out at two hydrogen ion concentrations, namely, p_H 7·7 and p_H 5·6, the two buffers being of the same ionic strength, 0·1. With this concentration of electrolytes, the pressures are not appreciably influenced by traces of carbon dioxide. After equilibrium had been attained, estimations were made of the concentration of the protein and, in certain cases, of the density of the protein and of the membrane potential, by a slight modification of the method of Loeb [1922].

The concentration of the protein was calculated from refractometric measurements [Adair and Robinson, 1930, 1] supported by Kjeldahl nitrogen determinations. The percentage of nitrogen in globin was calculated by deducting four g. molecules or 2525 g. of haematin, containing 224 g. nitrogen from 1 g. mol. or 67,000 g. of haemoglobin. The percentage of nitrogen in the dried haemoglobin of the ox is stated to be 17·7, but a reference to the original paper [Hüfner, 1887] showed that this figure is apparently based on a single analysis. It was deemed advisable to redetermine the nitrogen content by the method of Kjeldahl, and from the result obtained, 16·75 $\pm$ 0·15, it

was estimated that ox-globin contains 17·06 g. of nitrogen per 100 g. dry protein, and therefore the globin concentration is equal to the nitrogen multiplied by 5·862. The same factor has been used for sheep's globin. Schulz [1898] gives the value 16·89 % of nitrogen for horse-globin.

II. The stability of native globin.

According to Hill and Holden [1926] and Haurowitz and Waelsch [1929], globin is an unstable protein. We have made a number of tests to determine whether the degree of stability of the protein under the conditions of our experiments is sufficient for the purpose of osmometric measurements, in which the protein is equilibrated with buffer solutions at p_H 7·7 or p_H 5·6 for periods varying from 7 to 27 days at 0°.

(i) No diffusible protein breakdown products could be detected when the dialysates were tested by the xanthoproteic and the biuret reactions at the end of equilibration.

(ii) Haematin was added to the globin solutions after equilibration at p_H 7·7 and 5·6, and it was found by spectroscopic observations that in all cases the protein had retained the property, characteristic of native globin, of forming methaemoglobin, and subsequently reduced and oxy-haemoglobin on reduction and oxygenation. Since very small traces of native globin are required for this reaction, the spectroscopic tests are of only qualitative significance, as a proof that a certain amount of globin remains unchanged after equilibration.

(iii) Solutions of globin after equilibration at p_H 5·6 at 0° were brought to the isoelectric point by addition of alkali or phosphate buffer. Since native globin is soluble and para- and denatured globins are insoluble at the isoelectric point, the formation of a precipitate is a proof of alteration in the globin. The results for different solutions are recorded in Sections IV and V. It may be noted that in cases where experiments were continued for 18 to 28 days after the date of preparation, considerable amounts of precipitate were found, but in a number of experiments on dilute solutions, which were completed within 10 or 12 days, only traces of protein were precipitated. Attempts were made to estimate the amount of paraglobin formed by determining the total nitrogen and the soluble nitrogen after neutralisation, but the results of these analyses have not been recorded in the Tables because it is not yet known whether the precipitate formed is paraglobin only, or a mixture containing occluded native protein. In a few experiments, marked "much paraglobin" in Table III, about 70 % of the protein was apparently precipitated at the isoelectric point, but in other experiments the proportion precipitated varied from 0 to about 30 % of the total protein.

(iv) Further evidence concerning the stability of globin was obtained by osmometric experiments, in which the pressures were observed daily for periods from 7 to 25 days, and the osmometers were reset, in order to see if the

pressures returned to their original values. These experiments indicated that the stability of globin depends upon the species, the method of preparation, and the concentration of protein and of electrolytes. The best results were obtained with native globin from ox-blood at p_H 7·7. In Exp. 517, the pressure remained constant within 0·1 mm. for 7 days. At p_H 5·6 there were greater variations, probably owing to the transformation of native globin into paraglobin; the manometer readings diminished, instead of remaining constant, in experiments made with solutions containing more than 6 % of protein. The native globin of the horse is less stable; almost all the protein precipitated at p_H 7·7, and no estimate of osmotic pressure could be made, but at p_H 5·6 the pressures were constant within about 0·2 mm. Hg for a few days. A similar degree of constancy was observed in experiments on the paraglobin of the ox and the horse, unless the protein gelatinised, when the pressure fell more rapidly.

The reproducibility of the final pressures, obtained in a series of experiments with membranes of varying permeability and with different times for equilibration, is an additional criterion not only for the existence of osmotic equilibrium but also for the stability of the protein. The degree of reproducibility attainable in the case of dilute solutions of a fairly stable protein, such as serum-albumin, is illustrated in a series of 27 experiments in which the deviations from the mean pressure range from 0 to 4 %, recorded by Adair and Robinson [1930, 2]. In the series of 19 experiments with ox-globin at p_H 5·6, given in Table III, in 5 experiments the deviations from the mean pressure are less than 1 %; in 8 experiments, the deviations range from 1 to 4 %; in 4 experiments there are deviations of 4 to 10 %, and in 2 experiments from 10·5 to 13·1 %. It seems therefore that the preparation of ox-globin must have a certain degree of stability at p_H 5·6 as the deviations are not excessive, although they are considerably greater than those observed for serum-albumin. The measurement of the osmotic pressure of globin presents greater difficulties and the range of error is increased because the time available for obtaining a rigorous proof of equilibrium is limited by the fact that the protein may change into paraglobin.

III. The osmotic pressure of globin solutions at p_H 7·7.

Investigations of the osmotic pressure of globin at hydrogen ion concentrations varying from p_H 6·74 to p_H 8·26 are of special interest, as it has been shown by Roche [1930] that paraglobin and denatured globin are insoluble within this range. The pressures obtained should therefore be unaffected by such degradation products of globin.

Considerable advantage is obtained by the use of a buffer solution at approximately the isoelectric point of the protein, as at such a concentration of hydrogen ions the partial pressure due to the unequal distribution of diffusible ions, designated p_i, is very small, and the observed pressure is

approximately equal to p_p, the partial pressure of the protein ions. The isoelectric point of native globin has been determined by Roche [1929; 1930] by the method of cataphoresis, and found to be p_H 7·5 at 18°. Adair, Cordero and Shen [1929] have found that the p_H of mixtures of haemoglobin and bicarbonates increases from 0·014 to 0·018 if the temperature be diminished by 1°; it was inferred therefore that the isoelectric point of native globin should be about p_H 7·7 to p_H 7·8 at 0°, the temperature at which our osmotic experiments were carried out. This inference was supported by measurements of the membrane potential, recorded below.

The initial concentration of the buffer used was KH_2PO_4 0·00357 *M* and Na_2HPO_4 0·03214 *M*. The buffer value of phosphates is not very great at this hydrogen ion concentration, but previous experiments on haemoglobin have shown that if the ionic strength of the buffer be 0·1 or more, the observed osmotic pressures of dilute solutions of the protein are practically constant over a considerable range of p_H values.

In most of the experiments referred to below, in which no exceptional precautions were taken to exclude CO_2, the final percentage of phosphate ions in the bivalent form, calculated from hydrogen electrode measurements, was 85 % instead of 90 % as calculated from the original composition. Since one bivalent ion is equivalent to four univalent ions, the final value of the ionic strength of the buffers must have been from 0·1 to approximately 0·097.

Table I. *Native globin of the ox, osmotic pressures at* p_H *7·71–7·74 and 0°.*

Dialysate Na_2HPO_4, 0·03214 *M* + KH_2PO_4, 0·00357 *M*; ionic strength 0·10.

Preparation No.	Exp. No.	C	C_v	p	π_v	E
9 F	528	0·92	0·92	1·77	1·92	—
9 G	520	0·96	0·97	1·68	1·74	—
9 F	527	1·58	1·61	2·38	1·48	−0·04
7	496	1·58	1·61	2·42	1·50	—
9 F	526	1·72	1·75	3·00	1·72	—
9 G	519	1·91	1·94	2·91	1·50	—
9 J	529	2·12	2·16	3·23	1·50	−0·02
9 J	530	2·32	2·37	3·72	1·57	+0·03
9 G	518	2·77	2·85	3·89	1·37	−0·10
9 K	536	3·13	3·23	4·58	1·42	−0·15
7	495	3·42	3·53	4·46	1·27	−0·12
7	494	3·45	3·57	4·48	1·26	+0·3
9 G	516	3·94	4·09	5·22	1·28	—
9 G	517	4·04	4·20	5·26	1·26	−0·25
9 K	539	5·52	5·83	6·49	1·11	−0·42

C = g. dry protein per 100 cc. solution; C_v = g. dry protein per 100 cc. solvent; p = osmotic pressure in mm. Hg at 0°; $\pi_v = p/C_v$; E = membrane potential in millivolts.

The results obtained from a series of experiments on native globin solutions of concentrations ranging from 0·915 % to 5·52 % are given in Table I. Comparative experiments, carried out on carbon monoxide haemoglobin, made from the ox-haemoglobin solution that had been used for the preparations of globin numbered 9 F to 9 K are recorded in Table II.

Table II. *Carbon monoxide haemoglobin of the ox, osmotic pressures and membrane potentials at* p_H *7·75 and 0°.*

Dialysate Na_2HPO_4, 0·03214 *M* + KH_2PO_4, 0·00357 *M*, ionic strength 0·10.

Exp. No.	C	C_v	p	π_v	π_p	E observed	E calculated
549	0·89	0·89	2·35	2·63	2·62	—	−0·07
548	1·80	1·83	4·89	2·67	2·63	—	−0·14
547	3·01	3·10	8·82	2·85	2·79	−0·18	−0·24
546	4·95	5·19	15·02	2·90	2·81	−0·42	−0·40
545	8·57	9·34	29·62	3·18	3·01	−0·76	−0·72
544	14·26	16·55	58·30	3·53	3·23	−1·33	−1·27
543	16·51	19·62	75·40	3·84	3·48	−1·43	−1·50

Two methods for the tabulation of the protein concentrations have been adopted in Table I. Under the heading C, the results are given in g. per 100 cc. of solution; the chief advantages of this method are that C is directly proportional to the refractive index of the protein solution and the nitrogen content determined by volumetric analysis, and that it involves no speculations concerning the hydration of the protein. The corrected concentration C_v is calculated from measurements of C by formula (1).

$$C_v = C \times 100/(100 - V_1 C) \qquad \text{.....(1).}$$

V_1 equals the volume occupied by 1 g. of dry protein *plus* the water of hydration combined with it. In the case of haemoglobin, in which about 0·2 g. of water appears to combine with 1 g. of protein [Adair and Robinson, 1931], the value of V_1 is about 0·965, and for the purpose of provisional calculations, the same value has been used for globin. The corrected concentration, C_v, is of service in the theoretical interpretation of the osmotic pressures.

A number of measurements of the membrane potentials of globin and haemoglobin are recorded in Tables I and II. The potentials obtained with haemoglobin are directly proportional to the protein concentration, as shown by the agreement between the observed values of E and these calculated by the empirical formula $E = k_e C_v$, where k_e is a constant equal to − 0·077. The negative sign shows that under these conditions the protein is acting as an anion.

In the experiments on globin, the conditions were not favourable for accurate measurements of membrane potentials. The observed potentials were small, and the available evidence is consistent with the assumption that at p_H 7·7 the protein is not far from the isoelectric point.

IV. The osmotic pressures of native globin and paraglobin of the ox at p_H 5·6.

One of the objects of this work was to compare the osmotic pressures of native globin and of paraglobin under the same conditions. Such a comparison is not possible at the isoelectric point, because paraglobin is insoluble. On the other hand, it is not possible to use solvents that are very far from neutrality, because native globin is then converted into paraglobin.

The determinations made previously by Roche [1930] indicated that at p_H 5·6 the solubility of paraglobin should be sufficient for the purpose of osmotic pressure determinations, and preliminary investigations made in this work showed that although native globin tends to change into paraglobin at p_H 5·6, the rate of change over a period of 7 to 14 days is not excessive, for in many of the experiments only a small fraction of the total protein was precipitated, when, at the end of equilibration, alkali was added to raise the p_H from 5·6 to the isoelectric point.

A list of the experiments made on preparations of native protein has been given in Table III. In the case of the preparations numbered 6 and 7, it appears that the pressure concentration ratio $p/C_v = \pi_v$ is a constant equal to 4·58, over the range of pressures from 4 to 50 mm. Of the 14 determina-

Table III. *Native globin of the ox, osmotic pressures at* p_H *5·64 and 0°.*

Dialysate sodium acetate, 0·10 *M* + acetic acid, 0·0105 *M*.
Preparation 6: method of Hamsik; preparations 7 to 9 θ, method of Anson and Mirsky.

Preparation No.	Exp. No.	C	C_v	p	π_v	E	Test for paraglobin
6	483	3·09	3·19	14·62	4·59	—	None
6	481	1·53	1·56	7·02	4·51	—	Trace
6	480	1·49	1·51	6·88	4·57	—	None
6	485	1·06	1·07	4·46	4·18	—	,,
6	484	0·95	0·96	4·51	4·72	—	,,
6	482	0·86	0·87	3·96	4·60	—	,,
7	486	10·18	11·30	48·80	4·32	+3·35	Some
7	487	10·18	11·30	53·30	4·72	+3·35	,,
7	488	7·54	8·13	37·40	4·60	+2·55	,,
7	489	5·79	6·13	28·60	4·66	+1·85	,,
7	490	3·33	3·42	15·46	4·52	+1·05	Little
7	491	1·64	1·67	7·59	4·55	—	Trace
7	492	0·84	0·85	4·10	4·83	—	Little
7	493	0·80	0·81	4·25	5·26	—	Some
9 G	521	1·51	1·53	7·22	4·73	—	Much
9 G	522	1·01	1·02	4·86	4·75	+0·05	,,
9 B	525	2·50	2·56	10·76	4·21	+0·57	Some
9 JA	531	2·08	2·13	9·63	4·52	+0·40	Much
9 θ	538	1·46	1·48	6·06	4·10	+0·45	,,

tions, 10 come within about 3 % of 4·58, and 4 show larger deviations, ranging from 6 to 15 %, but it is doubtful whether any significance can be attached to these deviations, because it was deemed advisable to finish the series rapidly, owing to the risk of denaturation of globin at p_H 5·6, and it is possible that the time allotted for equilibration and for determinations of capillarity corrections was insufficient to exclude the risk of experimental errors.

In the cases of preparations 9 G and 9 JA, in which the protein was equilibrated with buffer at p_H 7·7 before it was brought to p_H 5·6, the values for π_v agree with 4·58 within the limits of experimental error, but in the cases of preparations 9 B and 9 θ, there are deviations of 8 and 10 % respectively, which are probably due to the presence of paraglobin. It is interesting to note that preparation 6, made by the method of Hamsik, gives the same result as the other preparations, made by the method of Anson and Mirsky.

Before discussing the theoretical significance of the formula $p = 4{\cdot}58\ C_v$ for the osmotic pressure of globin, it is necessary to estimate the pressure, symbolised p_i, due to the excess of diffusible ions inside the membrane at p_H 5·6.

In the case of ideal or infinitely dilute solutions, the pressure p_i can be calculated by Donnan's equations. A different method has been developed [Adair, 1929] and a formula (No. 2) has been obtained which can be applied to non-ideal systems in which the activities of the diffusible salts are kept constant at different protein concentrations by equilibration with buffer solutions of the same composition.

$$p_i = RT \int_0^u m_p n_p du \qquad \text{.....(2).}$$

p_i = the pressure due to the excess of diffusible ions inside the membrane, expressed in mm. of mercury.

$$RT = 22{\cdot}4 \times 760 = 17{,}033 \text{ at } 0°.$$
$$u = E \times (F/1000RT) = E/23{\cdot}5 \text{ at } 0°,$$

where E = the membrane potential in millivolts.

$m_p n_p$ = the equivalent concentration of the protein acetate (the product of the molar concentration m_p and the valence n_p) in g. equivalents per litre of solvent.

The estimation of the equivalent concentration $m_p n_p$ has been discussed by Adair and Robinson [1930, 2], who have suggested that if E be small, under certain conditions the effects due to deviations from the ideal solution

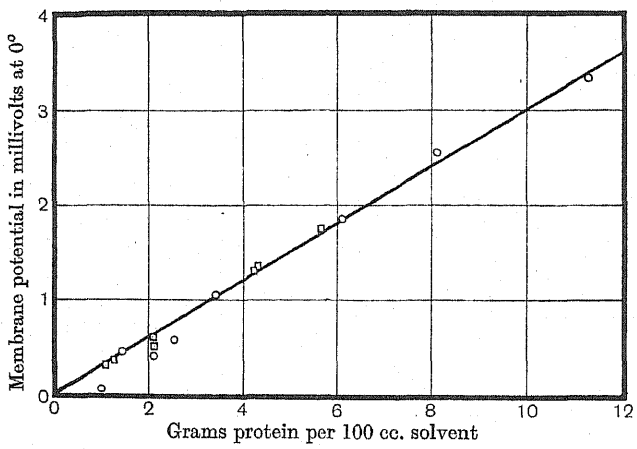


Fig. 1. Membrane potentials of globin solutions equilibrated with acetate buffer at p_H 5·64. Circles, native globin of ox. Squares, unfractionated globin and paraglobin.

laws may cancel out, and $m_p n_p$ may be equal to $-uJ$, where J is the sum of the concentrations of the ions in the dialysate, multiplied by the squares of their valencies. The ionic strength is equal to $\frac{1}{2}J$. A simple formula for p_i (No. 3) can be derived from formula (2), on the assumption that $m_p n_p = -uJ$.

This assumption is consistent with the experiments recorded in Table III and Fig. 1, which show that membrane potentials are directly proportional to the protein concentrations over the range investigated.

$$p_i = \tfrac{1}{2}RTJu^2 = 15{\cdot}4\,JE^2 = 15{\cdot}4\,Jk^2c_v^2 \qquad \text{.....(3).}$$

The term k in the formula numbered (3) is the ratio E/C_v, which must be a constant over the range of application of formula (3). In the observations recorded in Table III, $k = 0{\cdot}3$ and $J = 0{\cdot}2$.

It is probable that calculations of p_i based upon determinations of C_v and of the mean value of the ratio $E/C_v = k$ are more accurate than calculations based upon the individual values of the membrane potentials, which are subject to greater experimental errors than the measurements of protein concentration.

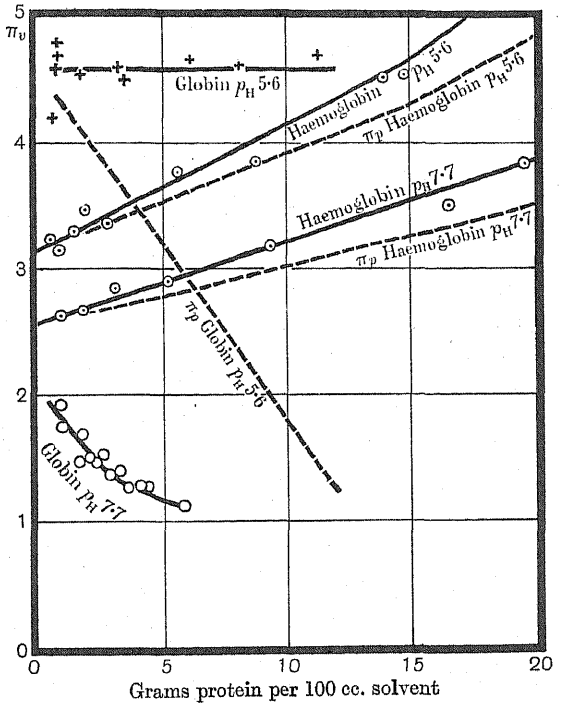


Fig. 2. Native globin and haemoglobin of ox. Pressure concentration ratios. Ordinate π_v and π_p in mm. of mercury at 0°. Abscissa C_v. Full lines, π_v; broken lines, π_p = ratio of partial pressure of protein ions and protein concentration. π_p is equal to π_v in solutions of globin at p_H 7·7.

The results recorded in Fig. 2 show that although the ratio π_v is practically constant, the ratio $\pi_p = p_p/C_v = (p - p_i)/C_v$ tends to diminish as the protein concentration is increased. This diminution is discussed in Section VII.

The results of measurements of the osmotic pressure of paraglobin are recorded in Table IV. The pressures observed are smaller than those given by native globin.

Table IV. *Unfractionated globin and paraglobin of the ox, osmotic pressures at* p_H *5·64 and 0°.*

Dialysate sodium acetate, 0·10 *M* + acetic acid 0·0105 *M*.

Preparations 1 and 2: unfractionated globin not dried before dialysis; preparation 4: unfractionated globin stored in dry state before dialysis; preparations 9 C and 9 L paraglobin.

Preparation No.	Exp. No.	C	C_v	p	π_v	E
1	364	1·25	1·27	4·51	3·57	+0·35
2	369	1·07	1·08	3·28	3·03	+0·30
4	391	2·05	2·09	6·81	3·26	+0·60
4	392	2·08	2·12	7·05	3·32	+0·5
4	389	4·06	4·23	11·60	2·75	+1·3
4	390	4·10	4·27	10·45	2·45	+1·35
9 C	523*	5·22	—	6·79	—	+1·35
9 C	524	2·58	2·65	4·56	1·72	—
9 L	537	5·38	5·68	9·61	1·69	+1·75

* Exp. 523, some protein in gelatinous form.

The osmotic pressures of preparations of unfractionated globin are given in Table IV, and it will be observed that the results are intermediate between those recorded for native globin and for the paraglobin separated by precipitation.

The osmotic pressures of fractionated native globin and paraglobin of the sheep have not yet been determined, but a preliminary series of measurements on unfractionated globin has been made, the results of which are recorded in Table V (prep. 3), in which the values of π_v diminished from 1·38 to

Table V. *Unfractionated globin of the sheep, osmotic pressures at* p_H *5·64 and 0°.*

Preparation No.	Exp. No.	C	C_v	p	π_v	α'
3	382	4·61	4·82	5·71	1·19	0·00185
3	383	2·28	2·33	2·89	1·24	0·00188
3	384	2·30	2·35	2·86	1·22	0·00187
3	385	3·35	3·47	4·28	1·23	0·00189
3	386	3·33	3·45	4·16	1·21	0·00189
3	387	1·15	1·16	1·56	1·34	0·00179
3	388	1·11	1·12	1·57	1·40	0·00187
5	433	3·31	3·42	5·70	1·67	0·00188

α' = the specific refraction increment (ref. Section VI).

1·17 as the protein concentration was increased. It appears that the unfractionated globin of the sheep gives lower osmotic pressures than any of the other forms of globin studied in this work.

For comparison with the experiments on globin, determinations of the osmotic pressure of haemoglobin at p_H 5·65 have been recorded in Table VI. The carbon monoxide and methaemoglobin solutions were prepared from the stock solution of haemoglobin used for the globin preparations numbered 9 G to 9 θ. The osmotic pressures are discussed in Section VII. The observed membrane potentials agree with those calculated by the empirical formula $E = kC_v$, given in the last column of Table VI, assuming that k is + 0·089 for CO-haemoglobin and + 0·119 for methaemoglobin.

Table VI. *Haemoglobin of the ox, osmotic pressure and membrane potentials at* p_H *5·65 and 0°.*

Dialysate sodium acetate, 0·10 *M* + acetic acid, 0·0105 *M*.
Nos. 540*, 541*, 542* methaemoglobin. All other experiments CO-haemoglobin.

No.	C	C_v	p	π_v	π_p	E observed	E calculated
533	0·58	0·59	1·89	3·25	3·21	—	+0·05
534	0·95	0·96	3·01	3·15	3·13	—	+0·09
514	1·44	1·46	4·80	3·30	3·27	—	+0·13
513	1·86	1·90	6·52	3·44	3·39	+0·10	+0·17
512	2·72	2·79	9·36	3·36	3·29	+0·28	+0·25
511	5·46	5·62	21·2	3·77	3·63	+0·63	+0·50
510	8·07	8·76	33·7	3·85	3·63	—	+0·80
509	12·34	14·00	63·4	4·53	4·18	+0·90	+1·25
535	12·97	14·82	67·5	4·55	4·19	+1·33	+1·32
515	15·38	18·05	92·8	5·14	4·69	+1·70	+1·61
508	17·88	21·60	120·2	5·56	5·03	+1·75	+1·93
532	20·90	26·20	161·9	6·18	5·53	+2·67	+2·34
540*	3·30	3·41	11·36	3·33	3·19	+0·32	+0·41
542*	3·39	3·50	12·10	3·46	3·31	+0·47	+0·42
541*	3·77	3·91	13·39	3·42	3·25	+0·47	+0·47

V. The osmotic pressures of native globin and paraglobin of the horse.

In order to compare the osmotic pressures of globins prepared from crystallised and from uncrystallised haemoglobins, experiments were carried out on the globin of the horse, the haemoglobin of which can be crystallised without difficulty.

In the preparation 8 *b*, referred to below, crystals of the carbon monoxide haemoglobin of the horse were obtained by the addition at 0° of an equal volume of saturated ammonium sulphate to a solution of haemoglobin which had been separated from the stroma by the addition of ether and salt. The mother-liquor was removed on a Büchner filter, and the crystals were redissolved and recrystallised and freed from impurities by dialysis.

Preparations of globin were made by the method of Anson and Mirsky from this material and from uncrystallised haemoglobin. Unfortunately, the globin of the horse appears to be less stable than that of the ox, and most of the protein precipitated during the removal of ammonium sulphate by dialysis. Attempts to measure the osmotic pressure at p_H 7·7 were moreover unsuccessful, as most of the protein precipitated. Six determinations obtained at p_H 5·64 are recorded in Table VII.

The solutions were so dilute that the difference between the concentration C and the corrected concentration C_v is within the limits of experimental error. The calculation of the protein concentration from nitrogen determinations is not recorded, because the amounts of material available were insufficient for adequate analyses, and provisionally all the calculations have been based on refractometric measurements, assuming that the specific refraction increment for horse-globin is 0·00183, the same as that determined for ox-globin (ref.

Table VII. *Native globin and paraglobin of the horse, osmotic pressures at* p_H *5·6 and 0°.*

Preparation 8 *a*, native globin from uncrystallised haemoglobin; preparation 8 *b*, native globin from recrystallised haemoglobin; preparation 8 *c* paraglobin.

Preparation No.	Exp. No.	*C*	*p*	*E*	π^*	Test for paraglobin
8 *b*	497	0·68	3·94	+0·23	5·8	Trace
8 *b*	498	0·68	3·85	+0·36	5·7	Some
8 *b*	499	0·45	2·80	—	6·2	Trace
8 *b*	500	0·47	2·66	+0·28	5·7	Some
8 *a*	501	1·11	6·43	+0·50	5·8	Much
8 *a*	502	0·60	3·63	—	6·1	Trace
8 *c*	505	1·89	5·00	+0·85	2·7	—
8 *c*	506	0·94	2·54	—	2·7	—
8 *c*	507	0·95	2·72	—	2·9	—

$$\pi^* = p/C.$$

Section VI). In the solutions containing less than 0·5 g. of protein per 100 cc., the error in the refractometric determinations may be about 5 %.

It appears that the mean value for π is 5·88, whereas the value obtained for ox-globin is about 4·58. The difference is about four times as great as the maximum observed deviation from the mean value 5·88, and it seems probable that the difference is a real phenomenon. In the second place, it appears that the osmotic pressures of the globins prepared from recrystallised and from uncrystallised haemoglobin are the same, within the limits of experimental error. As in the case of ox-globins, the osmotic pressure of the paraglobin of the horse is less than half the pressure given by the native globin.

VI. A comparison of the densities and the specific refraction increments of globin and haemoglobin.

In a number of the experiments made in this research, the measurements of osmotic pressure were supplemented by determinations of the specific refraction increments and the densities of the protein solutions.

The measurements of densities are recorded in Table VIII. It seems that for both proteins, the density of the protein solution, D', is directly proportional to the concentration C expressed in g. per 100 cc. of solution, as stated in formula (4).

$$D' = D'' + Ck_d \qquad \text{.....(4).}$$

D'' = density of the dialysate; k_d = an empirical coefficient. This formula has been used in calculations of osmotic pressures of dilute solutions, in which the observed heights of the columns in cm. of protein solution must be reduced to mm. mercury. The values of k_d have been calculated to five places, but the fifth figure is uncertain, and for the purposes of this work it is sufficient to determine k_d approximately to four places. The values of k_d for haemoglobin and for globin in both the solvents used are 0·00256 $\pm$ 0·0001. It is not yet possible to say whether the slight deviations from 0·00256 are of any significance.

Table VIII. *Densities of solutions of globin and haemoglobin of ox, equilibrated with buffer mixtures at 0°.*

Buffer 7·7. Na_2HPO_4, 0·03214 *M* + KH_2PO_4, 0·00357 *M*.
Buffer 5·6. CH_3COONa, 0·10 *M* + CH_3COOH, 0·0105 *M*.

No.	Protein	p_H	Density of dialysate D''	Density of protein D'	C	k_d
544	CO-haemoglobin	7·7	1·0047	1·0404	14·26	0·00250
543	,,	7·7	1·0050	1·0467	16·51	0·00253
515	,,	5·6	1·0045	1·0435	15·41	0·00253
508	,,	5·6	1·0045	1·0496	17·89	0·00252
532	,,	5·6	1·0046	1·0561	20·90	0·00247
540	Methaemoglobin	5·6	1·0043	1·0126	3·30	0·00250
527	Globin	7·7	1·0051	1·0093	1·58	0·00266
518	,,	7·7	1·0046	1·0119	2·77	0·00263
516	,,	7·7	1·0053	1·0152	3·95	0·00252
525	,,	5·6	1·0045	1·0108	2·49	0·00250
488	,,	5·6	1·0041	1·0238	7·54	0·00261

Measurements of the specific refraction increments of haemoglobin and of globin are recorded in Table IX. The numbers of the experiments have been stated and other data concerning the protein concentration and conditions in these experiments are given in Tables I, II, III and VI. In accordance with previous work on the serum-proteins [Adair and Robinson, 1930, 1] it was found that the value of α', defined by formula (5), is independent of the protein concentration, C, expressed in g. per 100 cc. of solution.

$$R' - R'' = \alpha' C \qquad \text{.....(5).}$$

R' = refractive index of protein solution; R'' = refractive index of dialysate. All dialyses were carried out at 0° but the measurements of R' and R'' were made at about 15°.

Table IX. *Specific refraction increments of carbon monoxide haemoglobin and of globin of the ox, equilibrated with phosphate and acetate buffers at 0°.*

Haemoglobin			Globin p_H 7·7		Globin p_H 5·6	
Exp. No	Buffer	α'	Exp. No.	α'	Exp. No.	α'
543	7·7	0·001955	516	0·00180	481	0·00183
544	7·7	0·001935	526	0·00179	486	0·00182
545	7·7	0·001965	527	0·00179	488	0·00185
546	7·7	0·001941	528	0·00176	489	0·00182
508	5·6	0·001955	530	0·00175	525	0·00181
513	5·6	0·001988	536	0·00178	531	0·00194
515	5·6	0·001960	539	0·00178	538	0·00189
534	5·6	0·001960	—	—	—	—

Theoretically, there may be appreciable differences between α' and α, the specific refraction increment of the protein in water, because $R' - R''$ is affected by the unequal distribution of salts across the membrane. The refraction increments of the salts in the dialysates were estimated by deducting the refractive index of water, and the results ranged from 0·0011 to 0·0012 for the acetate buffer at p_H 5·6 and from 0·00095 to 0·00099 for the phosphate buffer at p_H 7·7. In the case of globin at p_H 5·6, the membrane potentials

indicate that there is an appreciable excess of acetate ions inside the membrane, and it is possible that the refraction due to the diffusible ions is greater than 0·0012. In the systems containing haemoglobin and globin at p_H 7·7 the potentials are small, and it is unlikely that there is much difference between the refractions due to salts on both sides of the membrane.

In the case of human oxyhaemoglobin, α is 0·001942 [Stoddard and Adair, 1923], and we have obtained a similar value of α, namely 0·001952 for the haemoglobin of the ox. The values of α' recorded in Table IX agree with 0·00195 within the limits of experimental error.

In the case of the native globin of the ox at p_H 7·7, the mean value of α' is 0·00178. The earlier experiments on native globin at p_H 5·6 gave a mean value for α' of approximately 0·00183, but some of the experiments equilibrated for longer periods gave higher values and in the case of unfractionated globin (preparation 4, Table III) the following figures were obtained: 0·00190, 0·00188, 0·00188, 0·00190. It seems possible that the higher values are due to the presence of paraglobin, but the problem requires further investigation.

The values of α' for native globin at p_H 7·7 are similar to those obtained for egg-albumin and serum-albumin. Haurowitz and Waelsch [1929] have published lower values for globin, ranging from 0·00146 to 0·00154, but from the account of their experiment it seems unlikely that the concentration term C in their paper is the same as C in formula (5), which is calculated from nitrogen determinations, assuming that anhydrous globin contains 17·06 % of nitrogen. It is not improbable that the powdered globin used by Haurowitz and Waelsch was a hydrate containing less than 17 % of nitrogen. Schulz's [1898] preparations of globin, dried at a higher temperature (100°) contained about 16·89 % of nitrogen. Robertson [1912] prepared globin by Schulz's method and obtained values for α of 0·00169 $\pm$ 0·00005 for his purest preparation.

VII. Discussion.

In the formation of globin from haemoglobin by the action of acid acetone, 1 g. mol. or 67,000 g. of haemoglobin yields 4 g. mols or about 2525 g. haematin ($C_{34}H_{30}O_4N_4FeOH$) and the difference of about 64,475 must represent the mass of globin. The ideal osmotic pressure of a substance of this molecular weight is given by the formula $p/C_v = 2{\cdot}64$ at 0°.

The observed pressure ratios differ from 2·64. In discussing these differences, there are three factors which must be considered. These factors are represented by the symbols p_i, g_p and M in the general formula for the osmotic pressure of a protein, restated below.

$$p = p_i + p_p = p_i + RTg_p.10C_v/M \qquad \text{.....(6).}$$

p = observed osmotic pressure; p_i = ion pressure difference; p_p = partial osmotic pressure of protein ions; RT, the product of the pressure and volume of an ideal gas, = 22·4 × 760 = 17,033 at 0°; g_p = the osmotic coefficient of the protein ions, which represents the effects of attractive and repulsive forces

causing deviations from the ideal solution laws, M = the molecular weight, or the mean molecular weight if the protein is a mixture of aggregates. M may be a variable affected by the protein concentration if the aggregates can dissociate into smaller particles in accordance with the law of mass action.

The factor p_i can be calculated from membrane potential measurements, as described in Section IV, and the number of variables reduced to 2 by the calculation of the ratio π_p defined below.

$$\pi_p = p_p/C_p = 10RTg_p/M \qquad \text{.....(7).}$$

The results of calculations of π_p for the native globin of the ox are recorded in Fig. 2, in which π_p is plotted against C_p.

For comparison with the experiments on globin, a number of determinations of the osmotic pressure of haemoglobin in the same solvents, at p_H 7·7 and at p_H 5·6, have been made using carbon monoxide haemoglobin and methaemoglobin prepared from the stock solution of ox-haemoglobin utilised in the preparation of globins numbered 9 B to 9 K.

The osmotic pressures of CO-haemoglobin at p_H 7·7 may be considered first. Fig. 2 shows that π_v is about 2·6 in dilute solutions, and it increases as the protein concentration is increased. In both respects, this curve resembles the results obtained for the haemoglobins of the horse, the sheep, and of man, in solvents with p_H values ranging from 6·8 to 8·0, and with salt concentrations ranging from 0·01 to 0·23 mols per litre, recorded in previous papers [Adair, 1924; 1928].

It is almost certain that in the systems referred to above, the molecular weights of the proteins are constant, and that the changes in π_v are due to variations in the osmotic coefficients. If this condition is fulfilled it is legitimate to extrapolate the curve to determine the value of π_v when C_v is 0, as described by Adair and Robinson [1930, 2], and from the result obtained, namely 2·55 mm., it appears that the molecular weight of haemoglobin is about 67,000 in the case of the haemoglobin of the horse, sheep and man and the preparation of ox-haemoglobin used in this work.

The measurements on CO-haemoglobin and on methaemoglobin at p_H 5·6 show that π_v increases with increasing protein concentrations, an observation which proves that g_p must increase under these conditions, for any changes in aggregation which may occur tend to diminish π_p. In addition, the measurements indicate that π_p approximates to 3·15 in dilute solutions. Theoretically an effect of this order could be obtained if a certain proportion, not exceeding about 24 %, of the molecules dissociate into smaller units at p_H 5·6[1]. Svedberg and Nichols [1927] have shown that at p_H 5·4, the diffusion constant and the sedimentation velocity of haemoglobin differ by about 20 % from the normal

[1] Spectroscopic observations, for help with which we are greatly indebted to Prof. D. Keilin, revealed no denatured derivatives of haemoglobin in the material equilibrated for about 2 weeks at p_H 5·6 (in experiments numbered 540–542) but traces could be detected in material stored for longer periods.

values; their measurements being made on the CO-haemoglobin of the horse, at about 30°.

The results recorded in Fig. 2 show that there are remarkable differences between the osmotic pressures of globin and of haemoglobin. The osmotic pressure of globin is much more sensitive to changes in p_H, and the ratio π_p diminishes as the protein concentration is increased, even in the isoelectric region where π_p is equal to π_v.

Theoretically, the diminution in π_p must be due to a diminution of the osmotic coefficient, or a polymerisation of the protein, or to both of these factors acting in the same direction. There are two arguments in favour of the hypothesis that changes in polymerisation play an important part in the diminution of π_p. Firstly, this hypothesis agrees with the observation that, in dilute solutions, π_p at p_H 5·6 is two or three times as great as at p_H 7·7, for it is not improbable that an increase in acidity should favour the dissociation of the larger particles. Secondly, it appears to be a general rule that in the region of the isoelectric point, the osmotic coefficients increase rather than diminish. Since no exceptions to this rule have been found in previous investigations on the osmotic coefficients of the proteins, it seems reasonable to attribute the fall in π_p at the isoelectric point to changes in polymerisation rather than to a fall in the osmotic coefficient.

The arguments in favour of changes in the osmotic coefficient or of aggregation are more evenly balanced in the case of the ionised globin at p_H 5·6. The measurements of membrane potentials show that globin is more highly charged than haemoglobin, and according to the inter-ionic attraction theory of Debye and Hückel [1923] the osmotic coefficient should be diminished by an increase in the concentration of protein ions of high valency. On the other hand, the fact that the osmotic coefficient of haemoglobin in the same solvent fails to diminish is evidence in favour of the aggregation hypothesis. Nevertheless, as globin is more highly charged than haemoglobin at p_H 5·6 the diminution in π_p cannot be regarded as a definite proof of aggregation changes.

The calculation of the molecular weight of globin is complicated by the possibility that the protein undergoes polymerisation. The methods for the elimination of the effects due to the osmotic coefficient by extrapolation [Adair and Robinson, 1930, 2] cannot be applied to a system in which both M and g_p vary with the protein concentration and it will be seen that no attempt has been made to extrapolate the values of π_p for globin in Fig. 2. Although exact values cannot be obtained, rough estimates of the value of M for globin may throw light on the changes in aggregation which take place, and it is probable, though not certain, that in some of the systems studied in this work, the "uncorrected molecular weight" M/g, calculated by formula (7), is not greatly different from the corrected value. The results for native ox-globin at p_H 7·7 are given in Table X.

If M be a function of the protein concentration, the globin must be a mixture of polymerides. Svedberg and his colleagues have shown that there

Table X. *Effect of protein concentration on the uncorrected value of M for ox-globin at* p_H *7·7. (Subject to corrections of unknown magnitude.)*

(g. per 100 cc. solvent)	1·0	2·0	3·0	4·0	5·0	6·0
M (uncorrected)	94,000	110,000	123,000	139,000	150,000	155,000

are mixtures of aggregates in certain proteins, including their preparations of eu- and pseudo-globulins [Svedberg and Sjøgren, 1930; Mutzenbecher, 1931]. Svedberg [1930] has included globin in a list of proteins which are polydisperse. It is not improbable that the variations demonstrated by the centrifugal method are mainly due to the existence of simple physical mixtures of aggregates. Variations in *M* demonstrated by osmotic methods cannot be attributed to the existence of a simple physical mixture, and therefore it seems probable that in solutions of globin there is a dynamic equilibrium between the larger polymerides and the products of their dissociation.

The interpretation of the results obtained at p_H 5·6 presents greater difficulties. The measurements on native globins of the ox and the horse show that the ratio π_v is practically independent of the protein concentration over a considerable range. Since π_v and π_p approximate in dilute solutions, some provisional calculations of the value of *M* in dilute solutions have been made by using the observed values of π_v for comparisons with the results obtained for paraglobin.

Table XI. *Comparison of uncorrected molecular weights of different globins and paraglobins at* p_H *5·6 and approximately the same protein concentration (about 1 %).*

Ox native globin	37,000
Horse native globin	29,000
Ox paraglobin	99,000
Horse paraglobin	63,000
Ox unfractionated globin	52,000
Sheep unfractionated globin	125,000

The values of *M* for native globin are about one-half of the molecular weight of haemoglobin, but no conclusions can be drawn from this observation because *M* may vary with the protein concentration. It is clear that the degree of polymerisation is lower in native globin than in paraglobin or in unfractionated globin, and it may be that there is some correlation between the tendency for paraglobin to form larger aggregates and the fact that it can be precipitated more easily than native globin.

Summary.

The stability of globin at 0° in solutions buffered by phosphates and acetates has been investigated. In general globin is much less stable than haemoglobin, but preparations of the native globin of the ox have been made by the methods of Hamsik and of Anson and Mirsky, which give reproducible results in osmotic pressure measurements. The globin of the horse appears to be less stable than that of the ox.

In the case of ox-globin, the relationship between osmotic pressure and protein concentration has been studied at the isoelectric point, p_H 7·7, and at p_H 5·6, and for comparison with globin, experiments have been made on ox-haemoglobin, equilibrated with the same buffer mixtures. The partial pressure of the protein and the pressure due to the unequal distribution of ions have been estimated from measurements of membrane potential.

There is a striking difference between results obtained from globin and haemoglobin. In the case of globin, the ratio of partial pressure of protein and its concentration, π_v, tends to diminish as the protein concentration is increased. It is unlikely that this decrease is wholly due to deviations from the ideal solution laws, particularly in the case of isoelectric globin, and it seems probable that there is a dynamic equilibrium between polymerides of different sizes in solutions of globin, so that the average molecular weight is a function of the concentration of the protein, of hydrogen ion concentration and of other variables. At p_H 7·7, the mean molecular weight may be greater than that of haemoglobin.

Comparison of measurements of the osmotic pressure of unfractionated globin and of paraglobin with that of native globin at the same hydrogen ion concentration (p_H 5·6) indicates that paraglobin is more highly aggregated than native globin.

Preliminary investigations have been made on the osmotic pressures of the globins of the horse and the sheep.

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CCXV. THE CHEMISTRY OF THE WHITE ROTS OF WOOD.

III. THE EFFECT ON WOOD SUBSTANCE OF *GANODERMA APPLANATUM* (PERS.) PAT., *FOMES FOMENTARIUS* (LINN.) FR., *POLYPORUS ADUSTUS* (WILLD.) FR., *PLEUROTUS OSTREATUS* (JACQ.) FR., *ARMILLARIA MELLEA* (VAHL.) FR., *TRAMETES PINI* (BROT.) FR., AND *POLYSTICTUS ABIETINUS* (DICKS.) FR.

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In two previous communications [Campbell, 1930; 1931] experimental data have been adduced to indicate that white rot fungi are in no sense uniform with respect to the order in which they attack the principal components of wood. In the present investigation a further series has been examined with a view to determining whether the white rots can be classified into groups on the basis of their chemical action—individual members which attack wood in the same manner being included in the same group.

Experimental.

The experimental procedure and analytical methods were similar in all respects to those used in previous work except in the case of the decay caused by *A. mellea* which will be discussed later. The fungi to be studied were divided into three groups as follows:

1. *G. applanatum, F. fomentarius, P. adustus* and *P. ostreatus.*

The material to be decayed by this group of fungi consisted of a well-mixed sample of fresh, air-dried 60–80 mesh beech wood flour. Prior to inoculation each portion of wood flour was submitted to the usual sterilisation treatment, and the decayed wood was analysed after washing free from acid with cold water and drying at 105°.

The effect of 1 % NaOH at 100° was determined as before on both sound and decayed wood. The analytical results which are expressed as percentages by weight of the original oven-dry wood are recorded in Table I.

Table I. *The effect of a series of white rots on the chemical composition of beech wood, together with the effect of decay on the alkali-solubility of the residual wood substance.*

(Results expressed as percentages by weight of original oven-dry wood.)

		Decayed by:			
	Original wood	*Ganoderma applanatum*	*Fomes fomentarius*	*Polyporus adustus*	*Pleurotus ostreatus*
Before extraction with 1 % NaOH					
Duration of decay	—	2 months	4 months	4 months	4 months
Loss due to decay *plus* cold water washing	—	10·61	15·75	10·50	8·79
Cold water-soluble	1·03	1·32	1·03	0·89	0·45
Hot water-soluble	1·49	2·65	2·40	2·33	1·29
1 % NaOH-soluble	—	—	—	—	—
Cellulose	59·52	55·34	50·70	53·08	55·29
Lignin	20·54	16·81	16·00	18·03	18·60
Methoxyl	5·82	5·03	4·67	5·02	4·41
Total pentosans	22·28	19·46	18·40	19·76	19·86
Pentosans in cellulose	12·65	11·93	9·94	10·60	11·36
Pentosans not in cellulose	9·63	7·53	8·46	9·16	8·50
After extraction with 1 % NaOH for 1 hour at 100°					
1 % NaOH-soluble	14·57	19·73	18·33	18·75	17·00
Cellulose	58·49	47·12	46·9	50·52	49·99
Lignin	18·96	13·04	12·14	13·26	16·01
Methoxyl	4·66	3·46	3·44	3·71	2·39
Total pentosans	17·07	14·15	12·07	14·28	14·92
Pentosans in cellulose	11·75	7·83	8·51	10·04	9·42
Pentosans not in cellulose	5·32	6·32	3·56	4·24	5·50

In the samples inoculated with *G. applanatum* and *F. fomentarius* moderately large, tough wefts of mycelium formed on the surface of the decaying wood. In each case the weft was removed in a single sheet, carefully washed free from adhering particles of wood and weighed separately after drying at 105°. From 130·32 g. (oven-dry) of original wood decayed by *G. applanatum* 2·76 g. or 2·12 % of mycelium was obtained, while in the case of *F. fomentarius* 3·93 g. or 3·02 % was obtained from the same weight of wood. In the case of *P. adustus* and *P. ostreatus* the surface growth of mycelium was almost negligible in amount.

2. Heart rot of cherry wood (*Prunus avium* L.) caused by *Armillaria mellea*.

In a previous communication [Campbell, 1931] the effect of *A. mellea* on beech wood was described in some detail. At the stage of decay which was examined, however, the loss in weight was only 8·66 % and no attack on lignin was observed. Since these results were published, material in the form of an air-dry disc of cherry wood showing pronounced heart-rot caused by the same fungus has become available, and this has been examined with a view to gaining information regarding the more advanced stages of decay. In order to obtain an approximate measure of the loss in dry weight sustained by the central portion of the disc during decay it was necessary in the first instance to

determine the specific gravity of both sound and decayed wood. Eight specimens measuring approximately 1·5 × 1·5 × 5 cm. were cut at random from the outer sound heartwood of the disc and four samples were taken from the relatively small decayed central portion. It was considered advisable to determine the volumes of the specimens without wetting them and the author is indebted to Mr E. D. van Rest for drawing his attention to a paper by Hartmann, Westmont and Morgan [1926] from which it was possible to evolve a simple method of determining the volumes of wood samples by means of the displacement of sand. In the paper referred to it is stated that the bulk volume of a specimen of any porous material may be calculated from the difference in volume of sand displaced by the specimen and a metallic standard of approximately the same size and shape. It is shown that when this difference is small it is possible to determine the volume of the specimen with the same degree of precision as it is possible to measure the metallic standard. The fine sand employed in the present experiments was freed from organic matter, sifted to obtain 80–100 mesh material thoroughly washed and dried. The procedure employed was essentially the same as that indicated by Hartmann, Westmont and Morgan [1926] except that the operations were carried out on a much smaller scale. The sand was held in a filter-funnel to the lower end of which was fitted a short length of rubber tubing provided with a spring clip. A small cylindrical glass vessel served to retain the wood specimens and the steel standard, which latter measured 1·48 × 1·48 × 5 cm. at 15°.

It was found possible, with practice, to duplicate the weight of annular sand round either the standard or test pieces to within 0·2 g. in the majority of cases. With sand of bulk sp. gr. 1·455, 0·2 g. corresponds to a volume of 0·137 cc., so that, by accepting in each case the average of the two most closely approximating weights of annular sand it was considered that this method of determining the volumes of test pieces gave sufficiently accurate results.

After the air-dry volumes had been obtained the air-dry and oven-dry weights of each sample were determined. The results of the specific gravity determinations are given in Table II.

After conversion into wood flour of 60–80 mesh the sound and decayed materials were analysed in the usual manner (Table III). In view of the slight divergences in the moisture contents of the sound and decayed samples (Table II), the approximate loss in weight sustained by the wood which was decayed cannot be directly assessed by means of the true specific gravity figures but rather by comparing the averages of the quotients obtained in both series by dividing the dry weight of each sample by its air-dry volume. It can then be assumed, by neglecting the probable effect of partial decay on the swelling properties of wood and the normal slight variation in specific gravity found in all woods in passing from the centre of the tree outwards, that at any given temperature and relative humidity a given volume of the sound cherry heartwood contains more wood substance than the same volume of the

Table II. *Comparison of specific gravity of sound cherry heartwood with that of cherry heartwood of the same tree decayed by* A. mellea.

Specimen No.	Volume in cc. (air-dry)	Weight in g. (air-dry)	Weight in g. (oven-dry)	Moisture content % of oven-dry weight	Specific gravity Wt. (air-dry) / Vol. (air-dry)	Wt. (oven-dry) / Vol. (oven-dry)
	Sound wood					
1	10·657	7·361	6·555	12·3	0·691	0·615
2	6·038	4·528	4·029	12·46	0·750	0·667
3	8·873	5·469	4·862	12·46	0·616	0·548
4	10·149	6·945	6·185	12·28	0·684	0·609
5	11·867	7·448	6·675	11·59	0·628	0·562
6	10·763	7·042	6·304	11·71	0·654	0·586
7	11·647	7·718	6·897	11·91	0·663	0·592
8	9·49	6·519	5·812	12·17	0·687	0·612
				Average	0·672	0·599
			Decayed wood			
1	12·409	5·735	5·059	13·38	0·462	0·408
2	14·583	7·743	6·819	13·21	0·531	0·468
3	8·763	5·278	4·654	13·38	0·602	0·531
4	7·513	3·228	2·843	13·55	0·430	0·378
				Average	0·506	0·446

decayed wood in the proportion of approximately 1·34 : 1, or, alternatively that during decay the central portion of the disc has lost approximately 25 % of its oven-dry weight. By employing this latter figure the analytical data for the decayed wood can be expressed as percentages of the oven-dry weight of the original wood (Table III).

Table III. *The effect of heart-rot caused by* A. mellea *on the wood of cherry* (Prunus avium *L.*).

	Original wood %	Decayed wood %	Decayed wood % sound wood
Loss in weight due to decay	—	—	25·00 (approx.)
Cold water-soluble	2·97	4·47	3·35
Hot water-soluble	6·49	7·21	5·41
1 % NaOH-soluble	26·44	29·15	21·86
Cellulose	53·74	53·74	40·30
Lignin	19·32	20·97	15·73
Total pentosans	25·75	27·59	20·69
Pentosans in cellulose	13·20	14·31	10·73
Pentosans not in cellulose	12·55	13·28	9·96

3. *Trametes pini* and *Polystictus abietinus.*

The effect of *T. pini* was observed on a sample of silver fir (Table IV). In this case insufficient material was available for a detailed examination of the effect of 1 % NaOH on the sound and decayed wood. In the case of the decay caused by *P. abietinus* the starting material was 60–80 mesh Norway spruce and in this case both decayed and sound wood were analysed before and after extraction with 1 % NaOH for 1 hour (Table V).

Table IV. *Analysis of 60–80 mesh silver fir wood before and after decay by* Trametes pini.

Results expressed as percentages by weight of original oven-dry wood.

	Original wood	Decayed wood
Duration of decay	—	17 months
Loss due to decay *plus* cold water washing	23·01	—
Cold water-soluble	1·02	0·92
Hot water-soluble	1·87	2·5
1 % NaOH-soluble	12·0	12·98
Cellulose	57·46	46·47
Lignin	29·35	22·87
Methoxyl	4·81	3·7
Total pentosans	9·41	6·76
Pentosans in cellulose	3·18	2·61
Pentosans not in cellulose	6·23	4·15

Table V. *The effect of* Polystictus abietinus *on the chemical composition of the wood of Norway spruce, together with the effect of the decay on the alkali-solubility of the residual wood substance.*

Results expressed as percentages by weight of original oven-dry wood.

	Before extraction with 1 % NaOH		After extraction with 1 % NaOH for 1 hour at 100°	
	Original wood	Decayed wood	Original wood	Decayed wood
Duration of decay	—	5 months	—	—
Loss due to decay *plus* cold water washing	—	6·79	—	—
Cold water-soluble	1·67	1·64	—	—
Hot water-soluble	2·77	4·7	—	—
1 % NaOH-soluble	—	—	13·54	20·27
Cellulose	60·54	55·08	55·72	51·08
Lignin	26·22	23·16	23·85	17·36
Methoxyl	4·7	3·98	4·17	2·92
Total pentosans	10·28	8·87	8·59	6·22
Pentosans in cellulose	4·12	3·73	4·16	3·59
Pentosans not in cellulose	6·16	5·14	4·43	2·63

Discussion of results.

The results in Table I reveal that each fungus in the group has attacked carbohydrates as well as lignin. Well-defined differences with regard to the proportions of each constituent attacked, however, are at once apparent. For instance, *G. applanatum* and *P. adustus* acting on wood taken from the same original sample have, over widely different times, brought about an equal loss in weight and yet the latter has caused a greater depletion of cellulose than the former. This is in part compensated for by the fact that *G. applanatum* has a more pronounced action on lignin. Further differences are observed in the manner in which these two fungi attack the furfuraldehyde-yielding complexes of the wood. *G. applanatum* produces a relatively greater amount of depletion of the pentosans not associated with the cellulose than of the pentosans in the cellulose, whereas the reverse is true in the case of *P. adustus*. In the case of *G. applanatum* 2·12 parts of the 10·61 % loss in weight caused by the decay can be directly accounted for by the formation of a weft of mycelium

on the surface of the decaying wood while only a negligible amount of surface mycelial growth was observed on the wood decayed by *P. adustus*. These considerations point to pronounced differences in the biological activities of the two fungi although in a general sense they have the same ultimate chemical action on wood.

Comparison of the wood samples decayed by *P. adustus* and *P. ostreatus* respectively for the same length of time reveals that the two fungi deplete lignin to approximately the same extent although *P. ostreatus* causes a smaller total percentage loss of wood substance. At the same time at the stages of decay examined cellulose appears to be more resistant to *P. ostreatus* than to *P. adustus*. Slight differences in the respective effects of the two fungi on the furfuraldehyde-yielding complexes are also observed despite the fact that the depletion of total pentosans is the same in both cases.

In Table I the greatest degree of decomposition of the beech wood has been brought about by *F. fomentarius* but the net chemical effect only differs from that of *G. applanatum* in a few particulars. Much more cellulose has been decomposed while the extent of lignin depletion is not much more than in the decay caused by *G. applanatum*. The attack on pentosans is slightly more severe and the pentosans in the cellulose suffer proportionately greater depletion than the pentosans not associated with the cellulose. *F. fomentarius* resembles *G. applanatum* in that a considerable amount of mycelium is laid down, 3·02 parts of the 15·75 % loss in weight of the original wood being accounted for in this way.

Further examination of the analytical data reveals that each of the four fungi under consideration brings about an increase in the alkali-solubility of the residual wood substance at the stages of decay examined, but reference to a group of data recorded by Hawley and Wise [1926, p. 298] shows clearly that for equal percentage losses of wood substance all the white rots examined so far induce much smaller increases in alkali-solubility than the brown rots. It has been indicated previously [Campbell, 1930; 1931] that herein lies one of the principal chemical bases of differentiation between the two types of wood decay. In brown rots increase in alkali-solubility is roughly proportional to the amount of cellulose depletion and does not reach a maximum until a comparatively late stage of decay. In the white rots under consideration there is no proportionality between alkali-solubility and loss of cellulose or any other constituent.

The data in Table III illustrate the type of difficulty which is likely to arise in attempting to compare sound wood with wood which has been decayed by a white rot where the loss in weight due to decay is not known. It is suggested, however, that the method here used to determine loss in weight is sufficiently accurate for the purpose of the investigation, since it serves to confirm a previous conclusion [Campbell, 1931] that the decay caused by *A. mellea* must on purely chemical grounds be regarded as a variety of white rot. When the analytical data for the decayed wood are calculated as per-

centages by weight of oven-dry sound wood, it is observed that a stage has been reached at which lignin is decomposed, although the constituent which has been most severely attacked is undoubtedly the cellulose. As in the early stages of decay the pentosans associated with the cellulose have suffered depletion as well as the pentosans not in the cellulose. Finally, a definite decrease in alkali-solubility has taken place. At the stage under review the decay caused by *A. mellea* is closely similar in its effects to other white rots which have been studied.

The results in Tables IV and V illustrate the respective effects of two white rots on gymnospermic woods. *Trametes pini* has figured largely in the literature on wood decay as a typical white rot fungus in that it has been supposed to confine its attack to the lignin of wood and to leave the cellulose unchanged. The data in Table IV indicate clearly that no such specific action takes place and that the white rot caused by *T. pini* is comparable in general with other white rots which have been studied. The results appear to agree with the findings of Hawley, Wiertelak and Richards [1930] with regard to the same fungus.

Thaysen and Bunker [1927, p. 129] have quoted evidence in support of the view that *P. abietinus* causes a rot in which cellulose is depleted in the early stages but in which lignin is not attacked until decay is well advanced. The data in Table V, however, do not bear this out. At the early stage of decay under investigation, the attack on lignin is proportionately greater than the attack on cellulose, and of the two groups of pentosans those which are not associated with the cellulose are more susceptible to attack. In this case there is a comparatively sharp rise in alkali-solubility as cellulose and more especially lignin become more soluble in alkali as a direct result of attack. Unlike most of the other examples studied the pentosans not in the cellulose are more soluble in alkali than those which are associated with the cellulose.

Conclusions.

Sufficient data have now been obtained to show that as a class the white rot fungi are not consistent in the manner of their attack on wood, for although none has yet been found to have a specific action on lignin there is no general uniformity with regard to either the order or proportion in which the major wood components are decomposed. The evidence suggests that the white rots should be subdivided on chemical grounds into the following three groups.

Group I.

White rots in which lignin and pentosans are attacked in the early stages and in which the incidence of the attack on the cellulose proper is delayed. Representative fungus: *Polystictus versicolor* [Campbell, 1930; Lutz, 1931].

Group II.

White rots in which cellulose and its associated pentosans are attacked in the early stages and in which the incidence of the attack on lignin and pentosans not associated with the cellulose is delayed. Representative fungus: *Armillaria mellea* [Campbell, 1931].

Group III.

White rots in the early stages of which both lignin and cellulose are attacked but in varying proportions. Representative fungi: *Polyporus hispidus* [Campbell, 1931], *Ganoderma applanatum*, *Polyporus adustus*, *Pleurotus ostreatus*, and *Polystictus abietinus*.

It is considered that an early stage of decay might reasonably be regarded as one at which the observed loss in weight calculated as a percentage of the oven-dry original wood is approximately between 5 and 10. There is, therefore, insufficient evidence on which to classify *F. fomentarius* and *T. pini*, although the probability is that they will be found to fall into Group III. From an examination of the data obtained by Falck and Haag [1927] with respect to *Fomes annosus* it appears that this fungus also belongs to Group III, since if the specific gravity figures recorded by these authors be used in the manner previously indicated to calculate the results for the decayed wood as percentages of the weight of oven-dry sound wood it can be seen that the cellulose content as well as the lignin content decreases in the early stages of decay. In the same way it can be shown that the percentage alkali-solubility begins to decrease after the loss in weight of the original wood exceeds 20 to 25 % of the oven-dry original wood.

It should not be overlooked that the mere fact that it is possible to subdivide the white rots on the lines indicated above cannot be taken to indicate that each fungus induces a set of chemical reactions peculiar to the group to which it belongs. It would obviously be difficult at very advanced stages of decay to differentiate between one white rot and another by chemical means, and it is therefore highly probable that the ultimate primary reactions involved are the same in all three groups but that the main variants are the incidence in point of time of the primary reactions, the course of secondary reactions, if any, and the rates of the several reactions. It has been pointed out elsewhere [Campbell, 1932] that the principal enzymic reactions which are generally associated with the decay of wood by fungi are hydrolysis and oxidation. In brown rots hydrolysis appears to be the only primary reaction [Campbell and Booth, 1929] but it is suggested that in white rots decomposition is brought about by the combined action of oxidation and hydrolysis. In the present state of knowledge it appears that it is only on this basis that the observed behaviour of white rot fungi can be explained. The different rates of attack on the major wood components must be due in large measure to differences in the incidence and extent of secretion of at least two kinds of

enzymes with widely differing propensities. This contention derives support from the observation of Nutman [1929] that *Polyporus hispidus* secretes both hydrolysing and oxidising enzymes, while according to Lutz [1931] the same holds true for *Polystictus versicolor*, but, in addition, this fungus is capable of inducing at least one secondary reaction which results in the formation of alcohol from the products of the primary hydrolysis.

Consideration of the effect of white rot fungi on the pentosans of wood presents some difficulty. In brown rots, for instance, it is a general rule that of the two categories of pentosans those which are associated with the cellulose are the more susceptible to attack, but in white rots it appears that sometimes the one category and sometimes the other suffers relatively greater depletion without any reference to the extent of depletion in either the cellulose proper or lignin. In view of the above suggestion that both oxidising and hydrolysing enzymes play a part in all white rots it is not improbable that the true effect of decay on the wood pentosans is to some extent masked, for on the one hand it is known that oxycellulose yields a certain amount of pentose on hydrolysis, and on the other hand there is the possibility in some cases of the formation during decay of insoluble by-products which yield furfuraldehyde on hydrolysis with hydrochloric acid.

There appear to be good grounds for the conclusion that in white rots the solubility of the decayed wood in alkali rises to a maximum at a much earlier stage of decomposition than in brown rots. In certain instances [Campbell, 1930; 1931] this has been ascribed to the fact that the pentosans in decayed wood which are not associated with the cellulose are less soluble in alkali than the corresponding complexes in sound wood, but it is obvious from the results of the present investigation that this is not true for all white rots, since here it has only been found to hold for beech wood decayed by *G. applanatum* and *P. ostreatus*. These results appear to confirm the suggestion made in the preceding paragraph concerning the formation of by-products during decay. It is therefore obvious, in view of the general effect observed on the total alkali-solubility of wood decayed by white rot fungi, that the solubility of some major constituent other than pentosans must be consistently affected. It has been shown [Campbell and Booth, 1929] that in brown rots hydrolysis has the direct effect of increasing the alkali-solubility of cellulose, and Bray [1924] has shown that in the same type of decay the increase in the total alkali-solubility of the residual wood is proportional to the loss in cellulose. The suggestion accordingly presents itself that in white rots the full effect of hydrolysis on the alkali-solubility of cellulose is either masked or partially inhibited by the action of oxidases.

Summary.

1. A detailed chemical examination of a further series of white rots of wood tends to strengthen a previous conclusion that fungi of the white rot type invariably decompose carbohydrates as well as lignin, although there is

no general uniformity with regard to either the order or proportion in which the several wood components are decomposed.

2. The white rots can be subdivided on chemical grounds into three distinct groups as follows.

(i) White rots in which lignin and pentosans are attacked in the early stages and in which the incidence of the attack on cellulose is delayed.

(ii) White rots in which cellulose with its associated pentosans is attacked in the early stages and in which the incidence of the attack on lignin and the pentosans not in the cellulose is delayed.

(iii) White rots in the early stages of which both lignin and cellulose are attacked but in varying proportions.

3. It is suggested that the ultimate primary reactions involved in all white rots are oxidation and hydrolysis and that the main variants on which the above subdivision depends are the incidence in point of time of the primary reactions, the course of secondary reactions if any, and the rates of the several reactions.

4. In white rots the total alkali-solubility of the decayed wood as a percentage of oven-dry sound wood rises to a maximum and begins to decline at a much earlier stage than in brown rots.

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CCXVI. A NOTE ON MOVEMENTS AND PRECIPITATIONS OF AN AMPHOTERIC COLLOID DUE TO AN ELECTRIC FIELD AND SLIGHT ELECTROLYSIS.

A MODIFIED HARDY'S EXPERIMENT SUITABLE FOR DEMONSTRATION PURPOSES.

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While observing microscopically the cataphoretic movement of a gelatin-protected silver colloid it was found:

(1) That the particles in the centre of the field migrated towards the anode.

(2) That a wave of particles emerged from each electrode.

(3) That these waves ultimately met near the centre of the field, where they precipitated.

Hardy [1899] described similar effects using, however, U-tubes and boiled egg-white solution. Our experiment presents the same phenomena, but is so simply performed as to be suitable for class work.

It is conducted in large cells, the walls of which are formed by two glass plates (selected flat lantern slides do well for this purpose). The individual particles, of course, cannot be observed by this method, but the essential facts relative to the electrical phenomena known to occur in amphoteric colloids can be observed or deduced.

The nature of the silver colloid.

The silver colloid we employed was prepared by Bredig's method in a menstruum of 0·5 % gelatin (Coignet's best) in water. No salts were added separately. A slight amount of electrolysis occurs in the experiment. After 10–20 minutes' sparking with about 5 amp. passing at 230 v. d.c., the fluid acquires a dark brown colour, due to the colloidal dispersion of the silver. The colloid so obtained should be centrifuged to remove the larger particles, after which it may be preserved for months if it be protected from the influence of light. On examination under the high power microscope, using dark ground

illumination and a red light, innumerable minute particles are seen. The particles are, of course, in active Brownian movement and are mostly smaller than 0·2 $m\mu$ in diameter. As before mentioned, these particles migrate towards the anode in an electric field, showing that they are negatively charged. The reaction of the colloid is slightly alkaline.

During the preparation of the colloid, in the manner described, each particle of silver is considered to acquire an envelope of gelatin. This envelope apparently imparts properties to the colloid which are essentially those of gelatin. From this point of view the phenomena of the experiment will be discussed. The silver nucleus acts in the macroscopic experiment as a visible indicator of the location of the particles.

Experimental.

The two lantern slides must be chemically clean. For this purpose they are treated with chromic acid, water and alcohol, the alcohol being removed by burning rather than wiping. The glass plates are kept 0·03 mm. apart by the insertion of platinum foil electrodes at the opposite sides of the cell. The cell is completely filled with the colloidal solution, care being taken to exclude all air bubbles. Excessive colloid is wiped away from the edges of the cell before sealing it with melted paraffin. The paraffin is applied by means of a hot glass or metal rod. The finished cell will have a light yellowish-brown colour. The electrodes are then connected to a source of electricity, with the results shown in the photographs (Plate VII, Figs. 1 to 5). These were taken at the times indicated without interrupting the experiment, panchromatic plates being used. The cell was placed on a glass platform and illuminated from below, the camera being placed overhead in the inverted position.

The points to be noted are the following.

(1) A wave of particles first appears emerging from the negative electrode soon after the current is applied. This indicates that the particles of the colloid have a negative charge. The photographs demonstrate that the wave which comes from the negative electrode is smooth and shows no signs of precipitation.

(2) After a short but perceptible lag a much denser wave is seen to emerge from the positive electrode. The lag in the appearance of the positive wave is due to the attraction exerted by the positive electrode on the negatively charged particles of the colloid. No repulsion of these particles by the positive electrode can occur until their charge has changed its sign. This is effected by the electrolytic production of acid at the positive electrode. As soon as this change of sign has occurred the particles tend to be repelled from the electrode and from each other. In the process of changing the sign of the charge, precipitation of the particles occurs and this precipitation accounts for the greater density of the positive wave. Moreover, this wave shows an

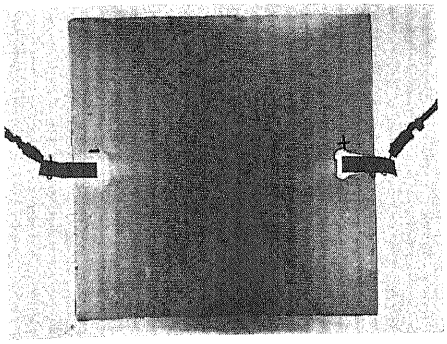


Fig. 1.

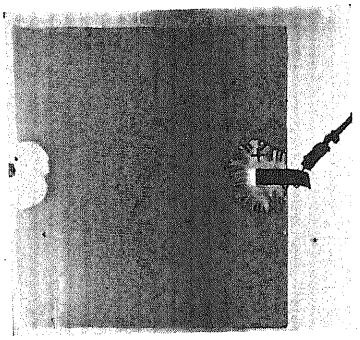


Fig. 2.

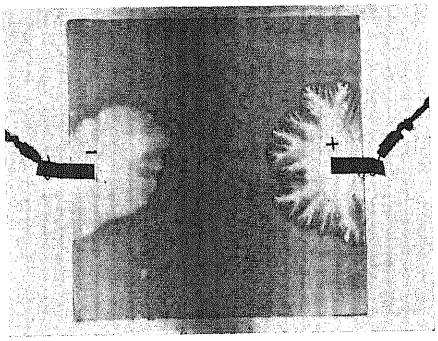


Fig. 3.

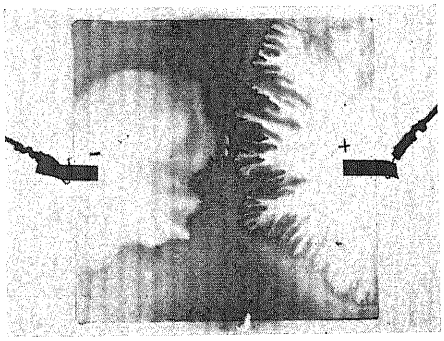


Fig. 4.

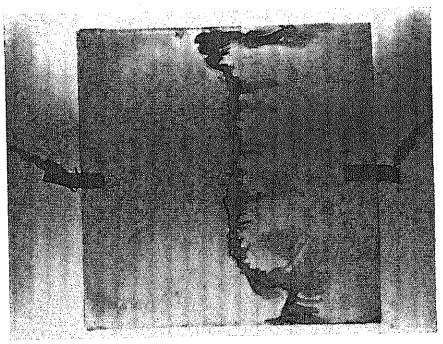


Fig. 5.

Figs. 1–5. Photographs of macroscopic experiment in progressive stages.

Fig. 1. Taken 3 minutes after application of 20 volts D.C.
Fig. 2. Taken after 10 minutes from beginning of experiment.
Fig. 3. Taken after 50 minutes from beginning of experiment.
Fig. 4. Taken after 150 minutes from beginning of experiment.
Fig. 5. Taken after 18 hours from beginning of experiment.

(The times stated are approximate.)

irregular convoluted formation, well illustrated by the photographs. The zone of precipitation in the positive wave is situated, in the early part of the experiment, near the positive electrode, but recedes from this electrode as the experiment proceeds. The approaching negative particles attracted by the anode are discharged, precipitated, reversed in sign and redispersed by the advancing acid wave. The positively charged particles behind the wave are strongly repelled by the positive electrode and tend to sweep before them the uncharged particles in the isoelectric state in the anode wave. Behind the dense anode wave proper we see, in the photograph, evidence of a smooth zone of dispersed particles, behind which is a clear acid zone. If the experiment be performed sufficiently slowly, the convolutions will not appear in the anode wave. On strongly increasing the potential across the cell, however, the positive wave immediately develops an undulating character. We believe that the precipitation of the particles is necessary before these undulations can occur. The change of sign of the particles apparently does not take place absolutely simultaneously, so that some of them are more repelled from the positive electrode than others. In this way, possibly, the convolutions may arise.

(3) In front of the positive wave there is a clear zone which is not present in advance of the negative wave.

(4) Ultimately the two waves meet and precipitate practically together near the centre of the cell. In this position a neutral zone is formed by the neutralisation of the acid and alkali of the respective waves. In this zone, naturally, the precipitation of the protein particles occurs. This is well shown in the photographs, where the positive and negative waves are united in a zone of common precipitation.

(5) If the current be stopped while the waves are advancing, they show a marked tendency to diffuse back again in the opposite directions to the directions in which they are travelling, showing that the charges of the electrodes are necessary for the movements of the waves. If the current be stopped after the common precipitation has occurred near the centre of the cell, then the precipitated waves remain unaltered for a considerable time.

(6) Slight evolution of gas, due to electrolysis, is seen to occur at each electrode.

A further experiment was carried out, in which strips of neutral litmus paper were placed in a cell of the macroscopic type. The paper should not contain an excess of alkali. This experiment showed that the acid and alkali waves preceded the visible positive and negative waves respectively for a small distance and showed the close relationship between these waves and the changes of reaction. It likewise showed that the zone of common precipitation occurred where the acid and alkali waves met. As the colloid waves recede from their respective electrodes, their velocities diminish. If the colloid be made slightly more alkaline by the addition of free alkali, the positive wave

has an increased lag in emerging and the zone of common precipitation is shifted towards the anode. On the other hand, if the colloid be slightly acidified, the positive wave emerges first and the appearances are reversed, the zone of common precipitation being now moved towards the cathode.

SUMMARY.

Photographs of a modified Hardy experiment are described, which demonstrate simply some of the electrical properties of amphoteric colloids. The experiment should be helpful for class demonstration purposes.

REFERENCE.

Hardy (1899). *J. Physiol.* **24**, 288.

CCXVII. A PRELIMINARY NOTE ON A PROLAMIN ISOLATED FROM A LEGUME SEED.

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(*Received September 21st, 1932.*)

The majority of the inhabitants of Egypt live on a variety of maize bread in the making of which a small proportion of the highly mucilaginous and bitter seeds of the legume known as fenugreek is added. Usually 2–5 % of fenugreek is used, but this small quantity has the advantage not only of conferring on the moist mixture better binding properties thus facilitating the production of the characteristic wafer-like loaf, but definitely makes the finished loaf more appetising.

While investigating the nutritional value of the protein mixture of this maize-fenugreek bread compared with that of ordinary wheaten bread, an alcohol-soluble protein was isolated from the dried powdered fenugreek seeds. This discovery appeared to be interesting since, according to Osborne [1924], alcohol-soluble proteins have so far been found only in the cereals.

Preparation.

After eliminating any cereal grains or other foreign materials, if present, the fenugreek seeds are ground in a clean strong laboratory mill, and the flour is subsequently extracted completely with ether. The ether extraction can be omitted, its only advantage being that it minimises loss in the later stages of the process as it gives a purer product.

The air-dried ether-extracted flour is now boiled with 70–72 % alcohol (1500 cc. to every 500 g.) on a water-bath. After 1 hour the hot contents of the flask are filtered rapidly through a Büchner funnel. The residue is extracted once more with the same strength of alcohol for another half hour, after which it is filtered hot as before.

On cooling, a yellowish-white precipitate begins to form and is allowed to settle in a covered vessel for 16 to 24 hours; the supernatant liquid is then carefully syphoned off and the moist precipitate centrifuged and subsequently washed with cold 70 % alcohol followed by 96 % until the washings are no longer yellow. The residue is then taken up in liquid phenol and again centrifuged. The greater part dissolves in the phenol giving a clear reddish-brown solution. The residue is treated two or three times more with phenol before it is discarded.

The phenol solutions are treated with 96 % alcohol until precipitation is maximum, and the precipitate is repeatedly washed with the same strength of alcohol until the last traces of phenol are removed. Many washings are required to effect this. Finally the precipitate is washed with absolute alcohol on a Büchner funnel and then with ether. The ether is allowed to evaporate and the dry material is powdered and kept in a desiccator over calcium chloride.

Properties.

The material thus obtained is an amorphous, tasteless and odourless white powder. It is very slightly soluble in distilled water, more soluble in dilute HCl but it dissolves freely in dilute alkali. Its solubility in alcohol is somewhat peculiar. It only dissolves in hot 70 % alcohol and separates on cooling; it is insoluble in 96 % alcohol. A solution of the material in dilute alkali gives the following reactions:

Biuret	Violet
Xanthoproteic	Positive
Millon's	,,
Rosenheim's tryptophan test	Very faint, on standing
Sulphur test	Positive
Molisch's	Negative

It is precipitated by picric acid and also by hydroferrocyanic acid and by half saturation with ammonium sulphate. Half saturation with magnesium sulphate produces a definite turbidity, but complete precipitation is only brought about by full saturation with this salt. The isoelectric point lies approximately at p_H 5·3.

The material contains 17·18 % N and 1·115 % S.

The yield is very small. Roughly, 0·2–0·5 g. of material is obtained from 100 g. of flour. The major part comes out in the first extraction, and there is, therefore, no necessity to extract more than twice with 70 % alcohol.

Conclusions.

The above description shows that the material isolated from fenugreek by treating its dry powdered seeds with boiling 70 % alcohol is a protein. This protein, for which the name "helbin" is proposed (from "Helba," Arabic for fenugreek), might be classed with the gliadins for, like the gliadins it is soluble in boiling 70 % alcohol and in phenol and is precipitated from its phenolic solution by the addition of ethyl alcohol, acetone, amyl alcohol or ether. It differs, however, from the gliadins of wheat and maize, for example, in being insoluble in cold 70 % alcohol. It is therefore of interest to determine the exact relations of helbin to the known gliadins. The anaphylactic reaction should be of great help in this connection and it is intended to study the serological reactions of this protein and to compare them with those of the other gliadins.

SUMMARY.

1. A compound soluble in hot 70 % alcohol and possessing the characters of a protein has been isolated from the seeds of the legume known as fenugreek.

2. It differs from the known gliadins in being insoluble in cold, but soluble in hot 70 % alcohol.

3. Like the gliadins, this protein is soluble in phenol and is precipitated from its phenolic solution by the addition of ethyl alcohol, ether, *etc.*

We wish to thank Prof. G. V. Anrep for his encouragement and interest in the work.

REFERENCE.

Osborne (1924). The vegetable proteins. (Longmans, London.)

CCXVIII. THE ANTISCORBUTIC VALUE OF FRESH LIME JUICE.

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(*Received September 21st, 1932.*)

Studying the antiscorbutic value of the lime, Chick, Hume and Skelton [1918] found that the majority of the preserved lime juices examined were valueless as a source of this important dietary factor. This valuable information satisfactorily explained the then growing lack of confidence in preserved lime juice as a protective or therapeutic agent against scurvy.

While determining the antiscorbutic content of preserved lime juice sent by the Department of Commerce and Industry, complete confirmation of the above observation was obtained. For none of the preserved juices subjected to the test contained any detectable quantity of the antiscorbutic principle.

With fresh lime juice, however, our results differed markedly from those reported by the same investigators. According to them, the minimum protective dose of fresh lime juice for a guinea-pig is 5 cc. daily, while in our case complete protection was attained with a much smaller dose. As a matter of fact our findings show clearly that there is no difference between the antiscorbutic content of the truly fresh ripe lime and that of the fresh lemon.

From the point of view of lime-growing countries like Egypt, this result is quite important both nutritionally and commercially. In this country fresh limes and lime juice are much preferred to lemons which are grown here on a limited scale. It follows that one may have to fall back on the lime in case of emergency, and it is, therefore, of great importance to know the exact place which must be assigned to this fruit among the antiscorbutic agents.

Before discussing the possible causes of the difference between our result and that observed by Chick and collaborators, a description of the experiments which led to this result will be given below.

Technique.

The diets. As a scurvy-producing diet the following ration was prepared: caseinogen 8, calcium carbonate 1·5, sodium chloride 1, oats 55, and bran to 100. This diet was given *ad lib.* supplemented with 0·5 cc. cod-liver oil daily per animal. When an adequate supply of the antiscorbutic was provided, the guinea-pigs grew at a good rate and appeared quite healthy. When, on the other hand, no antiscorbutic was given the animals exhibited, both during life

and at autopsy, a typical picture of scurvy. Death occurred 24 to 32 days from the commencement of the feeding and was always preceded by a decline in weight starting near the end of the second week.

For certain reasons a modification in the diet was introduced during the course of the experiment. The oats were replaced by an equivalent quantity of barley after proving that the change in no way influenced the scurvy-producing properties of the ration.

Experimental animals. Young guinea-pigs as obtained from a dealer were employed for the test. They were usually kept on greens and bread for 2 days before starting the feeding. Animals weighing 300–400 g. were, as a rule, selected, but in a very few cases guinea-pigs of weights slightly outside this range had to be used. The pigs were housed in capacious metal cages with wood shavings as bedding. Food and water were served *ad lib.* and the cod-liver oil and the juice under examination were fed by hand from a pipette.

It was found necessary to keep the mouth of the animals and the surrounding area of the face perfectly clean. When this was neglected stomatitis usually developed especially in those receiving a highly sugared juice. The food consumption of animals suffering from this disorder generally dropped, possibly from lack of appetite or from pain due to the inflamed condition of the mouth.

Preparation of the juice. The juice was prepared fresh daily from ripe fruits recently picked. Actually none of the fruits employed was older than 2 days at the outside. After being cut into halves, the limes were squeezed by hand into a muslin-covered funnel, and, to the strained juice, $CaCO_3$ and sugar were added in the proportion of 15 cc. juice, 0·5 g. $CaCO_3$ and 2 g. sugar and when all the sugar had dissolved, the volume was made up to 20 cc. with water. The mixture was then centrifuged and the supernatant fluid employed for the experiments.

The diagnosis of scurvy. A word may be said here about the plan adopted for the diagnosis of scurvy. It is intended to discuss only the *post mortem* findings. In severe cases, no difficulty is experienced in diagnosing the condition, for typical subcutaneous and intramuscular haemorrhages can be easily detected macroscopically in the characteristic sites. Similarly joint swellings, beading of the rib junctions, fragility of the long bones and looseness of the front teeth, which are constant accompaniments of the vascular changes, are also easily detectable. There are cases, however, which present a less complete picture of the disease. In these, haemorrhages are usually absent. Swellings of the joints and beading of the costochondral junctions, with or without fragile bones and loose teeth, are the only pathological changes to be seen. The diagnosis of these cases was not so easy, especially as similar bone changes have been ascribed to other deficiencies [Delf and Tozer, 1918]. Nevertheless these cases have been classed as mild scurvy, for they occurred only in animals receiving either an inadequate dose of the antiscorbutic or none at all. It will be remembered that each animal received by hand a daily dose of 0·5 cc. of tested cod-liver oil and that those getting an adequate supply

of the antiscorbutic principle were entirely free from any bone changes whatsoever. Another point, which seems to support the view that these bone changes are scorbutic in origin, is that in the same batch of guinea-pigs on the same insufficient dose of the antiscorbutic one encounters both types, that is, animals exhibiting at autopsy nothing but bone lesions and others showing haemorrhages as well.

The absence of scurvy was determined by the behaviour of the animals during life, especially as regards growth and general health, and by a careful study of the different systems after death.

A summary of the results obtained with fresh lime juice is embodied in Table I.

Table I. *Source of antiscorbutic principle. Fresh lime juice.*

Daily dose in cc.	Initial weight g.	Duration of exp. days	Result
0	250	25	Dead, scurvy
0	250	30	,,
0	300	32	,,
0	400	24	,,
0·5	350	46	,,
0·5	354	47	,,
0·5	370	49	,,
0·5	318	57	,,
0·75	440	66	Dead, no haemorrhages, fragile bones, enlarged knee joints and rib junctions
0·75	338	104	Killed, chronic scurvy, knee joints ankylosed
1·0	333	51	Dead, moderate scurvy
1·0	300	62	,,
1·0	340	93	Killed, mild scurvy, slight bone changes only
1·5	353	31	Dead, bronchopneumonia, no scurvy
1·5	358	93	Killed, no scurvy
1·5	343	44	Dead, liver abscess, no scurvy
1·5	320	93	Killed, no scurvy
1·5	350	93	,,
2	270	93	,,
2	300	93	,,
3·5	380	93	,,
3·5	250	93	,,

From Table I above, it can be clearly seen that the minimum protective dose of fresh lime juice, obtained as described earlier, is 1·5 cc., and that partial protection is conferred even by 0·5 cc. which was the smallest dose tried.

Having established this point, it became interesting to define the relation of the Egyptian lime to that of the West Indies, since the latter fruit was employed in most of the previous work on this problem. There is no doubt that Egyptian limes belong to the group of the *Citrus medica* var. *acida* and, as a matter of fact, are, to the layman, indistinguishable from the West Indian limes, few trees of which are grown in the gardens of the Horticultural Section of the Ministry of Agriculture.

Many explanations can be suggested to account for the difference between the antiscorbutic value of fresh lime juice as reported here and that found by

other workers. But it appears that the difference is most probably due to the fact that, in previous work, the lime juice employed was obtained from relatively much older fruits and was sometimes kept for as long as 2 months before examination. If this explanation is true, the juice of West Indian limes examined under the conditions detailed above, ought to have an antiscorbutic value similar to that of fresh Egyptian limes. The results reported by Davey [1921] for fresh lime juice make this conclusion highly probable. With a special sample of lime juice, the minimum protective dose appeared to be less than that reported earlier by Chick *et al.* [1918]. This result also supports the view that, in lime juice, the antiscorbutic principle undergoes deterioration at a much quicker rate than in the case of other citrus fruits such as the lemon.

Owing to the close of the season, it was not possible to obtain direct information on this point. It was, however, decided to make some use of the few West Indian limes left on the trees, for comparison with Egyptian limes on the lines of the work done by previous investigators. West Indian limes grown locally and Egyptian limes were subjected to examination at one and the same time. In the preparation of the juice, quite fresh and ripe fruits were used. These were cut into two and squeezed by hand; and to the juice, strained through muslin, sodium benzoate was added as a preservative. The juice was kept in a refrigerator throughout the experiment and fed as such to the animals from a burette. The results obtained are given in Table II.

Table II.

Source of antiscorbutic	Initial weight g.	Duration of exp. days	Result
Egyptian Limes			
1 cc. daily	303	41	Died, bone changes only
,,	358	42	Died, moderate scurvy
2 cc. daily	304	41	Died, bone changes only
,,	315	40	,,
3 cc. daily	350	45	,,
,,	270	51	Died, scurvy
West Indian Limes			
1 cc. daily	344	30	Died, moderate scurvy
,,	304	36	,,
2 cc. daily	360	35	,,
,,	364	56	Died, scurvy, severe
3 cc. daily	450	42	Died, moderate scurvy
,,	432	40	,,

Table II shows that the antiscorbutic principle undergoes deterioration in the juice obtained from Egyptian limes and kept in the refrigerator during the experiment, for 3 cc. failed to protect the animals against scurvy (*cf.* Table I). Compared with the West Indian lime juice, the animals on the Egyptian lime juice lived slightly longer. It is not possible, however, to attach any importance to this difference at present.

Discussion and Conclusions.

In determining the antiscorbutic value of fresh lime juice, Chick *et al.* [1918] employed imported limes which took 4 to 8 weeks in transit and the squeezed juice was fed for 2 months, that is, until a fresh consignment was received. Davey [1921], following a similar technique, arrived at practically the same conclusions regarding fresh lime juice.

In the first set of experiments presented above, the juice was prepared fresh daily from ripe fruits not more than 2 days gathered. If we agree that there is no essential difference between the lime grown in Egypt and that of other localities, the low antiscorbutic value reported previously for fresh lime juice can be ascribed to deterioration of the antiscorbutic principle. There seems to be no doubt that loss of this factor takes place at a much greater rate from lime juice than from lemon juice, and, as a result of the present work, one may conclude that loss starts in the intact fruit. Davey [1921] obtained slightly better results with green than with ripe limes. It thus appears that loss of the antiscorbutic principle commences with the process of ripening.

The experiments with the West Indian and Egyptian limes only show that in both juices the antiscorbutic principle is not stable for a long time. During the experimental period (approx. 2 months) deterioration was such that 3 cc. failed to protect against the disease.

Summary.

1. The antiscorbutic value of lime juice, prepared fresh daily from ripe Egyptian limes not more than 2 days gathered, was found to be 1·5 cc. per guinea-pig daily.

2. 3 cc. of "fresh" lime juice, prepared under the same conditions but kept in the refrigerator during the whole course of the experiment (approx. 2 months), failed to protect the guinea-pig against scurvy.

We wish to thank the Director of the Horticultural Section and Abdel Badei Eff. of the Gebel Asfar Farm for supplying us with most of the material. Our thanks are also due to Prof. G. V. Anrep for the interest he took in the work.

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Davey (1921). *Biochem. J.* **15**, 83.
Delf and Tozer (1918). *Biochem. J.* **12**, 416.

CCXIX. THE EFFECT OF HYDROLYSIS ON THE SUGARS OF NORMAL URINE.

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(*Received September 21st, 1932.*)

According to various investigators, the reducing power of normal urine may, after acid hydrolysis, follow one of three courses. It may either diminish, increase or remain unaltered. If we accept the view that normal urines usually contain variable quantities of ill-defined polysaccharides, the augmentation of the reducing power after hydrolysis can be satisfactorily explained. The frequency and magnitude of the rise suggest that, under normal conditions, the excretion of such polysaccharides cannot be very large, although the polysaccharide content of the urine can be appreciably increased by the ingestion of certain carbohydrates such as dextrin. Folin and Berglund [1922] definitely demonstrated the presence of erythrodextrin in the urine of a healthy individual after giving 200 g. of this material by mouth. In this particular case the increase in the reducing power after hydrolysis was very marked.

The explanation of the diminution of the reducing power after hydrolysis is, on the other hand, not so easy. Little is known about the nature of the non-carbohydrate reducing substances of urine apart from uric acid, urochrome and creatinine. There is reason to believe however that other reducing substances also occur. In the methods employed for the determination of the sugars of normal urine attempts are made, it is true, to remove these non-carbohydrate reducing substances but the inefficiency of these procedures is apparent when the methods are compared; no two methods agree when applied to one and the same urine. It follows that the diminution of the reducing power of normal urine, which sometimes occurs after hydrolysis, may be due to partial destruction of the sugars or of the non-carbohydrate reducing materials or of both. It is obvious that a diminution of the reducing power can be observed only in urines almost or completely free from polysaccharides. That such diminution is due to partial destruction of the sugars has often been suggested, but no direct experimental evidence, as far as we are aware, has been brought forward in support of this view.

Normal urine probably contains at least two sugars one of which is glucose whilst the other, which occurs in relatively greater proportion, has not yet been identified. This latter sugar will, for convenience, be called *iso*maltose, which name was given to it some time ago because of the occasional

similarity of its osazone to Fischer's *iso*maltosazone. Then, if we accept the view that the diminution of the reducing power is due to sugar destruction, it would be of interest to investigate the extent of the destruction as far as each sugar is concerned. This result should be more satisfactorily attained if some procedure were adopted by which the complete removal from the urine of the dextrin-like bodies and the other complex polysaccharides can be assured. The presence of these bodies must naturally disturb the result by masking the destructive action of the acid on the pre-existing sugars. Such mutually antagonistic effects must obtain in urines in which the total reduction is not altered by hydrolysis.

While it is possible to devise a means for the complete removal of the complex carbohydrates, the removal of sucrose is not so easy. Fortunately, however, sucrose is not frequently met with in the urine, besides which it is possible to limit the examination to urines of subjects known not to have partaken of foodstuffs containing this disaccharide.

Working on these lines and with the help of the osazone reaction it has been possible to demonstrate that hydrolysis by acid destroys a large part of the sugar called *iso*maltose while glucose seems to be unaffected.

Experimental.

All the urines investigated were collected from young healthy individuals and in every case the food taken by the subject was noted. About 150 samples were examined and these represented odd specimens as well as 24 hour collections.

Every sample was first treated with charcoal, this treatment being an essential step in the preparation of the osazones of the urine-sugars. However, it must be remembered that charcoal not only removes the substances which normally interfere with the proper crystallisation of the osazones, but unfortunately also removes a variable quantity of the urine-sugars. After having compared the action of five specimens of charcoal, it was found that the B.D.H. decolorising charcoal was most suitable, since it interfered least with the urine-sugars and gave a clear colourless filtrate.

The charcoal treatment consisted in adding 8 g. charcoal to 100 cc. urine and filtering after allowing the mixture to stand for 5 minutes. The clear colourless filtrate was employed for the determination of the reducing power and the preparation of the osazone both before and after hydrolysis.

The hydrolysis of the charcoal filtrate was done with the help of HCl in a manner similar to that recommended by Folin and Berglund [1922], 20 cc. were measured in a tube graduated for the purpose, 2 cc. 10 % HCl were then added and the contents heated in a boiling water-bath for 75 minutes. After thorough cooling, the hydrolysed filtrate was neutralised with a few drops of a strong solution of NaOH. At first phenolphthalein was used as an indicator but later its use was discontinued and the alkali was very carefully added till

the phosphate precipitate just began to form. Then water was added to bring the volume back to the 20 cc. mark.

The reducing power of the filtrate was determined by Shaffer and Hartman's method [1920] and in the preparation of the osazone the method described by Hassan [1928] was followed.

Changes in the reducing power.

As has been stated previously about 150 urines were examined and with the exception of three, all showed a marked diminution in the reducing power after hydrolysis. Table I gives the figures obtained in a few cases.

Table I. *Effect of hydrolysis on reducing power.*

Total reduction in mg. glucose per litre	
Before hydrolysis	After hydrolysis
412	350
322	150
266	124
120	34
370	350
158	132

The samples which showed an increase after the acid treatment constituted 2 % of the total and in all sucrose was proved to be present. As a matter of fact the subjects from whom these three specimens were obtained admitted partaking of meals containing pastries sweetened with cane sugar syrup.

The causes of this diminution may be numerous, but the present work has been limited to investigating the influence of the acid on the urine-sugars. It was thought that the osazone reaction would be helpful in this respect. This reaction is not only capable of demonstrating changes in the quantity of the sugars but it can also show, within reasonable limits, variations in their proportions. The osazones of the sugars of the charcoal-treated filtrate have therefore been prepared before and after hydrolysis and the products compared.

In order to have a clear idea of the following comparison, the reader is referred to the paper by Hassan [1928] in which a description of the osazones of the sugars of normal urine is given. There it is shown that these compounds are crystal mixtures formed of at least two components, and that, under suitable conditions, these crystal mixtures can be split into two osazones having distinct characters. Osazone I crystallises out in rosettes formed of broad flat needles, is soluble in hot water and melts at about 152°, and osazone II is identical with glucosazone.

On comparing the microscopic appearance of the two products, a striking difference was immediately noticed. The osazones before hydrolysis presented the usual crystal mixtures in which the proportions of the two osazones varied greatly. Those obtained after hydrolysis showed, on the other hand, a marked diminution in the broad needles and a tendency to aggregate in sheaf formations instead of the usual rosettes and in a large number of the samples the crystal mixtures were replaced by glucosazone sheaves (see Plate VIII).

This difference can only be due to one of two factors. Either hydrolysis increases the glucose content of the filtrate or destroys the urine-sugar which gives rise to osazone I. In the former case the glucosazone character will predominate by virtue of the increased concentration of glucose, while in the latter the destruction of the *iso*maltose will leave the field free to the pre-existing glucose. It can, however, be argued that the change in the crystal shapes might simply be due to changed conditions of crystallisation and not to an alteration in the proportions of the two sugars. This objection is quite reasonable because it is very unusual, when the osazones of the sugars of any one urine are recrystallised, to get the same crystal shapes. The crystal mixtures obtained on recrystallisation can assume a large variety of appearances depending on the rate of crystallisation and so forth, but it is always possible to see that in the new crystal mixtures the proportions of the two components remain practically the same. The difference cannot be due to the formation of new sugar because the charcoal employed adsorbs the dextrin-like bodies, at least in the concentration in which they occur normally. Moreover the result reported above, concerning the uniform diminution of the reducing power after hydrolysis, is in itself a proof that the charcoal-treated filtrates are free from compounds capable of giving rise to fresh reducing substances on hydrolysis; sucrose is, of course excluded.

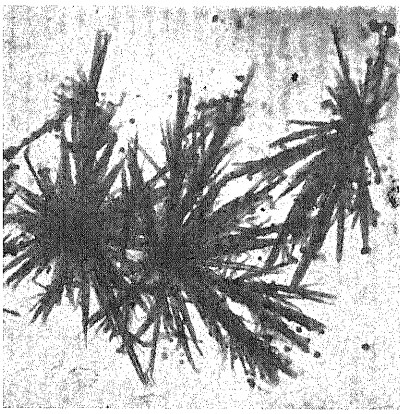
The experiment described above shows that the diminution in the reducing power and incidentally the change in the appearance of the crystal mixtures are probably due to destruction of *iso*maltose, *i.e.* the sugar which gives rise to osazone I. If this conclusion is correct then we should observe the following changes in the osazones of the hydrolysed filtrates.

(*a*) The osazone yield should be less.

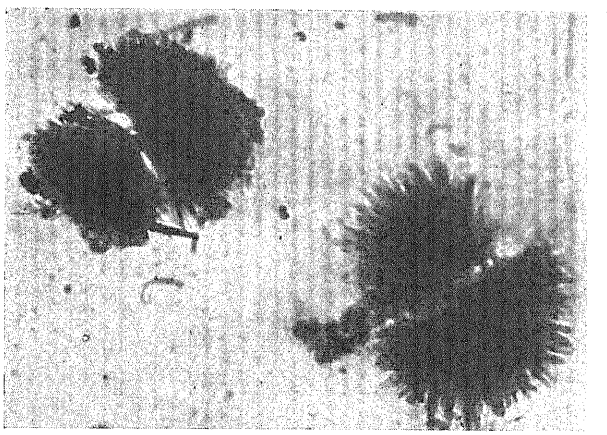
(*b*) There should be a rise in the M.P. of the crystal mixtures.

(*c*) The solubility of the crystal mixture should be less.

If destruction of the sugars is taking place during hydrolysis, one naturally expects to get less osazone formed after the acid treatment. To show that this is actually the case is by no means easy because of the difficulty of purifying the small yield without incurring great loss. After many trials we adopted the following procedure which, though it cannot give a pure product, is quite adequate for comparative purposes. The osazones were prepared under exactly similar conditions from the filtrates before and after hydrolysis. The precipitate was then filtered in a weighed Gooch crucible, washed with cold water and treated with as little chloroform as was necessary to wash away the oxidation products of the phenylhydrazine. The same volume of chloroform was used in both cases and the washing was stopped when the chloroform was free from colour. The crucible and contents were then dried to a constant weight. In all cases the osazone yield weighed less after hydrolysis (see Table II). The difference, however, did not always run parallel with that found for the reducing power. A possible explanation of this divergence will be discussed later on.



(*a*)



(*b*)

Osazones obtained from the same urine filtrate.
(*a*) Before, (*b*) after hydrolysis.

Table II. *Osazone yield.*

Weight of osazone in mg.	
Before hydrolysis	After hydrolysis
2·4	2·0
3·8	1·0
4·8	2·0
5·0	2·6
4·6	3·0

The melting-point and solubility.

Crystal mixtures vary in their M.P. and the variation depends on the proportion of each sugar present. Those containing more glucosazone would be expected to have a higher M.P. than those with relatively more of osazone I. If hydrolysis destroys the sugar giving rise to osazone I, that is, the osazone with the lower melting-point, a rise in the M.P. of the resulting osazone should follow. To prove this, an adequate quantity of the charcoal filtrates (50 to 100 cc.) was used for the preparation of the osazones and the melting-point of the crystals, purified by recrystallisation was determined. The following figures represent some of the results obtained.

Melting-point determinations	
Before hydrolysis	After hydrolysis
162°	181°
163°	186°
161°	180°

As would be expected the solubilities of the products exhibited unmistakable differences. In any one urine, the osazones obtained before hydrolysis were more soluble in hot water and in cold alcohol than those obtained after hydrolysis.

Discussion and conclusions.

Whether *iso*maltose or some other compound gives rise to osazone I, the parent substance seems to be slowly destroyed by acid hydrolysis, whilst glucose is unaffected under the same conditions. This assumption is supported by the microscopic appearance of the crystal mixtures in which an increase rather than a decrease of the glucosazone element is noted after hydrolysis. This increase is, however, only apparent, for the diminution in osazone I will not only give the glucosazone a chance to impress more of its own characters on the crystal mixtures but may, if the diminution is great, even allow it to form typical glucosazone. Moreover, subjecting an aqueous solution of glucose containing 40 mg. per 100 cc. to acid hydrolysis did not affect its reducing power. The destruction of *iso*maltose does not account, in many cases, for the total diminution in the reducing power and, as a matter of fact, there is no reason to believe that it can. Even in the charcoal-treated filtrate, the sugars are not the only reducing substances present. The lack of parallelism, referred to above, between the diminution in the reducing power and the

weight of the osazone found after hydrolysis points to the possible existence in the filtrate of non-carbohydrate reducing substances, and it is highly probable that some of these substances undergo destruction by acid as well.

SUMMARY.

1. The reducing powers of the charcoal-treated urines of about 150 healthy subjects were determined before and after hydrolysis with 10 % hydrochloric acid.

2. With the exception of three cases, in which sucrose occurred, a diminution in the reducing power was observed.

3. Evidence is given to show that this diminution is due, at least in part, to destruction of the so-called *iso*maltose occurring normally in urine.

We wish to thank Prof. G. V. Anrep for his encouragement and his interest in the work.

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CCXX. A SIMPLE METHOD FOR THE DETERMINATION OF THE MELTING POINT OF FATS AND ALLIED SUBSTANCES.

By MOHAMMED ABDOU ABBASSI.

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(*Received September 27th, 1932.*)

In the method to be described, use is made of the fact that fats and similar substances are, as a rule, bad conductors of electricity. A thin film of the substance whose M.P. is to be determined is interposed in the path of an electric current. Heat is then gently applied, and when the substance melts, the circuit is immediately completed and a bell is rung.

As seen from Fig. 1, the apparatus required is quite simple. The only part that needs description is the film-terminal (*A*). This is made by sealing the end of a piece of glass tubing, 0·5 cm. in diameter and about 20 cm. long, round a short length of platinum wire, the outer end of which should be filed flush with the surface of the sealed end. If allowed to protrude much beyond the outer surface, a large quantity of material would be required to coat the wire efficiently, with the result that the insulating film would be unduly thick. The flat cork (*C*) clamped to a retort stand, carries two holes to accommodate the film-terminal and the thermometer. The other parts of the apparatus comprise a Leclanché cell (*D*) or some other suitable source of current, an electric bell, a tall pyrex beaker three-quarters full of clean mercury, a tripod and a micro-burner.

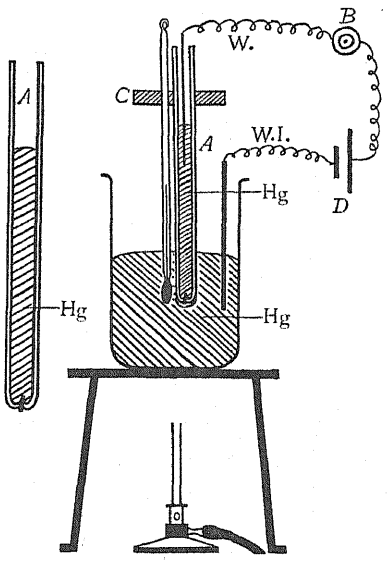


Fig. 1.

To carry out a determination, the substance whose melting-point is to be determined, is just melted in a clean dry test-tube and the clean dry bottom end of the film-terminal is dipped in the molten mass and quickly removed. The resulting film which need not cover more than the exposed end of the platinum wire and a small area of the glass surface round it, is then allowed

to solidify completely. The film-terminal is fixed in the cork in such a way that the end carrying the film stands close to, and level with the bulb of the thermometer. Both are then dipped in the cold mercury in the beaker to about 4 cm. from the surface and about this distance from the bottom. Clean mercury is now poured inside the film-terminal, and the wires from the cell (*W.* and *W.I.*) are brought into contact with the mercury in the film-terminal and that in the beaker as shown in the diagram. The beaker can now be heated slowly and the temperature at which the bell commences ringing is noted. This temperature represents the M.P. of the material for it is assumed that immediately melting takes place, the upthrust of the mercury pushes the molten mass up to the surface and contact is at once made between the mercury in the beaker and that in the film-terminal through the exposed platinum wire.

To ascertain the exact M.P., the experiment should be repeated at least twice, taking the usual precaution of heating slowly before the expected M.P. is reached. After each determination the end of the terminal is wiped with a soft cloth moistened with a proper solvent and then dried in alcohol followed by ether. It is advisable to have many such terminals ready for use in a clean covered jar.

The melting-points of a large number of suitable substances were repeatedly determined with encouraging results and the method was then compared with some of the methods already in use. The following table gives the melting-points of six samples of paraffin obtained by the present method (I) and by the help of Mason's melting-point apparatus (II).

Substance	I. M.P.	II. M.P.
A	55·4°	55·1°
B	59·3	59·3
C	47·9	47·8
D	43·9	43·9
E	48·7	47·8
F	51·5	51·1

Each result is the mean of at least three determinations and it will be seen that the figures in the two columns agree fairly closely.

This method is decidedly less laborious and certainly much cleaner than most of the methods in general use. The use of a stirrer is unnecessary because mercury is a very good conductor of heat, and tests have shown that, with slow heating, the temperatures taken at different places in the mercury-bath, are fairly even. The application of the method is naturally limited to substances capable of forming a thin compact non-conducting film, such as the fats, waxes, paraffin *etc.*

It is a great pleasure to me to thank Dr Ali Hassan for his help and encouragement.

CCXXI. HYDROGENLYASES.
II. SOME FACTORS CONCERNED IN THE PRODUCTION OF THE ENZYMES.

By JOHN YUDKIN.

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(*Received September 24th, 1932.*)

It has long been known that yeasts can be trained to ferment sugars upon which they are unable to act previous to training. The work of Dubourg [1899] on the training of yeasts to act upon cane sugar was criticised by Klöcker [1900]. But Dienert [1900] gave an account of some work on the acclimatisation of yeasts to galactose which is noteworthy. After growing yeasts on galactose, which, at the outset, they were not able to ferment, it was found that in circumstances where growth was impossible galactose was now fermented. The training was very rapid, 24 hours being sufficient to produce this property. The acclimatisation was lost only after growing on some other fermentable sugar for a long time. Yeasts which previously were unable to grow at all with galactose as the only carbon source, were acclimatised to galactose by growing in the presence of glucose and galactose. They were then able to grow with galactose as the sole carbon source.

Harden and Norris [1910] and Willstätter and Sobotka [1922] also trained yeasts to ferment galactose. The former showed that the press juice from acclimatised yeasts was able to ferment galactose. Abderhalden [1925] found that this property is not lost even if the yeast is grown for a long time on other carbon sources.

Similar work has been done on many other reactions induced by yeasts, moulds and bacteria. Thus Klotz [1906] trained a bacterium of the colon group to ferment lactose, and Twort [1907] was able to train *Bact. typhosus* to ferment lactose, and *Bact. dysenteriae* (Flexner) to ferment sucrose.

Karström [1930] described the adaptation of several species of bacteria to certain substrates. He concluded that bacterial enzymes may be classified as either constitutive or adaptive. The former are invariably present in the bacteria; the presence of the latter depends on the presence, in the medium on which the culture is grown, of the substrate upon which they act. Sometimes, the presence during growth of a certain substance inhibits the formation of an enzyme which is present in cultures grown on other media. For example, one of Karström's strains of *Bact. coli* contained saccharase when grown

on any sugar other than glucose, or when grown in the absence of sugar. Growth on glucose, however, gave a culture which had no saccharase.

It is necessary here to distinguish between "training" and "adaptation." First, it is clear that a given strain of bacterium can perform certain chemical reactions and cannot perform certain others, and that these properties normally remain constant. For example, a strain of *Bact. coli* growing on peptone and glucose ferments the glucose, but growing on peptone and sucrose does not ferment the sucrose. The phenomenon which has been called "training" consists in obtaining a strain which carries out a reaction which it was previously incapable of doing, by growing it for a large number of sub-cultures in a medium containing the new substrate. The new reaction then becomes a normal property of the strain. The phenomenon called "adaptation" is totally different, and is concerned with reactions which are already normal to the bacteria concerned. Of these reactions, some are catalysed by enzymes which are invariably present in the cells, on whatever medium they are grown; some by enzymes which are formed only when the cells are growing in the presence of the particular substrate. These two types are called respectively "constitutive" and "adaptive" enzymes. For example, one of Karström's strains of *Bact. coli* was able to ferment maltose immediately it was inoculated into a medium containing this sugar. It did not require to be trained to perform this reaction. However, a washed suspension of the bacteria grown in the absence of maltose was not able to ferment it, whilst a suspension of bacteria which had been grown in its presence did induce a fermentation, that is to say the enzyme is "adaptive." The training of organisms may proceed very rapidly as when Dienert [1900] acclimatised yeast to galactose in 24 hours. But once the organism has been trained, the newly acquired property is lost very slowly, if at all [Abderhalden, 1925]. On the other hand, an adaptive enzyme, which seems to be formed as a response to a chemical stimulus, is no longer produced when the stimulus is removed. This point is more fully discussed later.

Following the work of Karström, Stephenson and Stickland [1932] investigated the hydrogenlyases of bacteria, *i.e.* enzymes which liberate molecular hydrogen from formate, glucose, *etc.* It was found that washed suspensions of *Bact. coli* and *Bact. lactis aerogenes* possess formic hydrogenlyase when sodium formate is present in the medium on which they are grown, but not in its absence. The following work was undertaken with a view to determining further the factors concerned in the formation of these adaptive enzymes.

Experimental.

In all the experiments to be described, the bacteria in question were inoculated from an 8-hour culture in caseinogen digest broth into the medium concerned. The amount of sodium formate, glycerol or glucose added was always 0·5 %. Growth was allowed to continue for 15–18 hours (unless

otherwise stated) at 37°. The culture was washed twice with Ringer's solution by centrifuging and then made up in uniform suspension in Ringer's solution. The resulting preparation of "non-proliferating bacteria" was investigated as follows in the Barcroft manometer at 40° [Stephenson and Stickland, 1932]. 1 cc. of bacterial suspension and 1 cc. of $M/20$ phosphate buffer (p_H 7·0) were placed in each cup. In one cup was placed 1 cc. of the substrate, usually in $M/10$ solution, and in the other 1 cc. of water. The inner small cups were half filled with 40 % NaOH for absorption of carbon dioxide. The apparatus was twice evacuated and each time filled with nitrogen which had been passed over heated copper to remove small amounts of oxygen.

After equilibration readings were taken at intervals of 5 or 10 minutes.

In the case where gas is being evolved from glucose or glycerol, the rate is constant for a long time—certainly more than an hour. However, when formic hydrogenlyase is under investigation, the rate is rarely constant for longer than 20 minutes. The reason for this falling off is partly due to the alkalinity which soon manifests itself, and partly due to the fact that the formic hydrogenlyase has a low affinity [Stephenson and Stickland, 1932]. Thus the concentration of formate soon falls below the point where the enzyme is fully saturated.

The activity of the hydrogenlyases is expressed as Q_{H_2} (analogous to Warburg's Q_{O_2} for oxygen uptake), that is, it is the number of mm.^{3} of hydrogen evolved by 1 mg. dry weight of bacteria in 1 hour. The samples of the suspensions used were subjected to a nitrogen estimation by the micro-Kjeldahl method. Nitrogen determinations on dried bacteria (washed twice with distilled water) were also made and thus the dry weight of bacteria in any suspension could be arrived at by a nitrogen estimation. The dried bacteria were prepared by drying in a steam-oven to constant weight.

Nitrogen content of dried bacteria.

In order to determine whether the nitrogen content of bacteria varies according to the medium on which they are grown, a series of estimations was made which is summarised in Table I. The medium upon which the cells were grown is indicated in column 2. The broth used was a tryptic digest of caseinogen, containing about 3 % hydrolysed protein. The inorganic medium is that given by Stephenson [1930, p. 275]. Column 3 gives the conditions in which the bacteria were grown. "Roux" indicates that the culture was grown in Roux bottles lying flat, each containing 150 cc. of medium; "flask, aerated" means that the culture was grown in 500 cc. of medium in a litre flask with air bubbling through; "flask, not aerated" means that the preparations were grown similarly, but without air bubbling through.

It will be seen that the effect of the medium or of the growth conditions on the nitrogen content is at most very small. So also is the difference between the various organisms studied. Nicolle and Alilaire [1909], who estimated the nitrogen content of several species of bacteria, found that there

were "différences relativement faibles d'une bactérie à l'autre." Their value for *Bact. coli*, however, was 10·32 % N. They do not state how they dried their bacteria.

Table I. *Nitrogen content of dried bacteria.*

Bacterium	Medium	Conditions	N as % of dry weight
Bact. freundii	Broth formate glycerol	Roux	12·4
Bact. lactis aerogenes (strain 124)	Broth	Roux	13·3
	Broth formate	Roux	12·9
	Broth glucose	Roux	13·6
	Broth formate	Flask, aerated	13·0
	Broth glucose	Flask, not aerated	12·2
	Broth glucose	Flask, aerated	12·0
		Mean	12·8
Bact. dispar	Broth formate	Roux	12·2
	Broth glucose	Roux	13·6
		Mean	12·9
Bact. cloacae (strain 259)	Broth glucose	Roux	12·0
	Inorganic glucose	Roux	11·1
		Mean	11·5
Bact. cloacae (strain 402)	Broth glucose	Roux	13·2
	Broth glucose	Flask, aerated	12·1
	Broth glucose	Flask, aerated	11·9
	Broth glucose	Flask, not aerated	10·3
		Mean	11·9
Bact. coli	Broth	Flask, aerated	12·3
	Broth	Flask, aerated	12·5
	Broth	Flask, not aerated	11·9
	Broth formate	Flask, aerated	12·4
	Broth formate	Flask, aerated	12·0
	Broth formate	Flask, not aerated	12·2
	Broth glucose	Flask, aerated	12·3
	Broth glucose	Flask, not aerated	11·3
		Mean	12·1

Influence of growth conditions on the formation of the hydrogenlyases.

(i) *Bact. coli.* A summary of the results obtained with *Bact. coli* is found in Table II. It is there seen that the production of both hydrogenlyases studied is powerfully influenced by aeration. The condition when the culture is grown on broth formate or broth glucose in flasks is practically anaerobic owing to the rapid evolution of gas and to the small surface exposed. In these conditions the production of hydrogenlyases is at its maximum; with broth formate Q_{H_2} values of 190 and 76 are obtained for formic and glucose hydrogenlyase respectively, while with broth glucose these give Q_{H_2} values of 240 and 185. In Roux bottles, where a large surface is exposed, less enzyme is formed—with broth formate the Q_{H_2} values fall to 52 and 48, and with broth glucose to 215 and 120. With air passing through the medium, no hydrogenlyases are formed, except some glucose hydrogenlyase with broth glucose.

It is seen from Table II that aerobiosis in the formate medium results in an increase in alkalinity. This is explained by the increased oxidation of the formate in aerobic conditions. It might be thought that the decreased amount

Table II. *Hydrogenlyases of* Bact. coli.

Medium	Conditions	Final p_H	Formic hydrogenlyase: mm.³ of H_2 per mg. N	Formic hydrogenlyase: Q_{H_2}	Glucose hydrogenlyase: mm.³ of H_2 per mg. N	Glucose hydrogenlyase: Q_{H_2}
Broth	Anaerobic	7·5	290	35	400	48
,,	F.N.A.	7·4	0	0	Small	Small
,,	R.	8·0	0	0	100	12
,,	F.A.	8·3	0	0	0	0
Broth formate	F.N.A.	7·6	1580	190	630	76
,,	R.	8·0	425	52	400	48
,,	F.A.	8·6	0	0	0	0
Broth glucose	F.N.A.	5·4	1970	240	1530	185
,,	R.	5·0	1780	215	980	120
,,	F.A.	5·1	0	0	370	45
Broth glycerol	R.	—	510	62	310	38
Inorg. glucose	F.N.A.	7·0	0	0	0	0
,,	R.	—	0	0	0	0
Inorg. glucose chalk	R.	—	0	0	0	0
Inorg. glycerol	R.	—	0	0	0	0
Inorg. glycerol chalk	R.	—	0	0	0	0

R. = Roux. F.A. = Flask, aerated. F.N.A. = Flask, not aerated.

of the enzymes in these last experiments was due to the concomitant alkalinity. However, aeration in the case of the glucose medium also results in diminished hydrogenlyase activity. Here there is a production of acid, but the different degrees of aerobiosis cause little difference in the final p_H. Increased aerobiosis in this medium again however results in decreased hydrogenlyase activity. It seems, therefore, that the amount of enzyme produced varies inversely with the amount of air present during growth.

Apart from aeration, the formation of formic hydrogenlyase is seen to be most powerfully influenced by the presence of formate. A small amount is formed in strictly anaerobic conditions in broth alone ($Q_{H_2} = 35$) but as formic acid always occurs during the bacterial decomposition of amino-acids, this requires no special explanation. Glucose and glycerol also favour the production of the formic hydrogenlyase, the former to a greater and the latter to a less extent than formate ($Q_{H_2} = 240$ and 62 respectively). Both these substances are, however, known to be decomposed by *Bact. coli* with the formation of formic acid.

Formate also favours the production of glucose hydrogenlyase ($Q_{H_2} = 76$) but not so markedly as glucose itself ($Q_{H_2} = 185$).

Thus formate and glucose in the medium both result in the formation of both hydrogenlyases. The fact that the presence of glucose involves also the presence of formate, accounts for the crossways action in one direction. The production of the glucose enzyme by formate may be due to some characteristic common to the formic and glucose molecules.

The estimation of the Q_{H_2} of the organism grown on an inorganic medium shows that the washed suspensions of such cultures are lacking in both the enzymes. This result was unexpected in view of the fact that hydrogen is formed by *Bact. coli* while growing on an inorganic medium in the presence

of glucose. The reason for this result is not clear. It may be that the absence of the enzyme in the washed suspension is due to the absence of some stabilising substance which is present in the broth.

(ii) *Bact. lactis aerogenes*. The results for two strains of this bacterium are shown in Table III. In calculating the Q_{H_2} values for strain 418, the rate

Table III. *Hydrogenlyases of* Bact. lactis aerogenes.

Medium	Conditions	Final p_H	Formic hydrogenlyase: mm.³ of H_2 per mg. N	Formic hydrogenlyase: Q_{H_2}	Glucose hydrogenlyase: mm.³ of H_2 per mg. N	Glucose hydrogenlyase: Q_{H_2}
Strain 124						
Broth	R.	7·5	0	0	0	0
Broth formate	F.N.A.	7·3	2100	270	440	57
,,	R.	—	0	0	0	0
,,	F.A.	7·8	0	0	0	0
Broth glucose	F.N.A.	5·6	Very small	Very small	Very small	Very small
,,	R.	7·1	1800	230	100–440	13–57
,,	R.	—	1600	205	200–610	26–77
,,	F.A.	7·1	17	2	9	1
Inorg. glucose	F.N.A.	6·0	400	50	400	50
,,	R.	6·3	350	45	500	65
Strain 418						
Broth	R.	7·5	0	0	0	0
Broth formate	F.N.A.	7·4	3650	465	500	72
,,	R.	7·6	0	0	0	0
,,	F.A.	7·8	0	0	0	0
Broth glucose	F.N.A.	5·3	0	0	0	0
,,	R.	7·0	4300	550	1400	180
,,	R.	7·1	5500	700	640	82
,,	F.A.	7·1	0	0	400	50
Inorg. glucose	R.	6·0	0	0	1750	225
,,	R.	—	0	0	950	120

R. = Roux. F.A. = Flask, aerated. F.N.A. = Flask, not aerated.

per mg. N was multiplied by the nitrogen content of strain 124. It was found impossible to determine the nitrogen content of strain 418 owing to difficulty in centrifuging. The bacteria came down moderately well when Ringer's solution was used for washing, but when distilled water was used in order to get a preparation of dried bacteria, the cells did not come down even after an hour's centrifuging. Since bacteria of different species have such small differences in their nitrogen content, it is very improbable that there is any appreciable difference between two strains of the same species.

The two strains give exactly similar results. When grown on broth alone, the enzymes are not produced. On broth formate, the bacteria form the enzymes only when little air is present, showing in this respect a greater sensitivity than *Bact. coli*. In Roux bottles, or aerated in a flask, the cultures do not produce the enzymes. The maximum amount of enzyme formed in the case of broth glucose occurs when the culture is grown in moderately aerobic conditions. The very small amounts of the enzymes formed by strain 124, and the absence of the enzymes from strain 418, when the cells are grown in a flask and not aerated, are probably due to the production of acid in these

conditions. The presence of air ensures more complete oxidation of the glucose, and consequently less acid production.

The glucose hydrogenlyase of a suspension of strain 124 which is grown on a broth glucose medium in Roux bottles, shows an initial low output of hydrogen. This increases after about 30 minutes to a new constant rate.

In striking contrast to *Bact. coli*, when grown on an inorganic medium *Bact. lactis aerogenes* produces quite appreciable amounts of the enzymes. Strain 124 forms both hydrogenlyases whilst strain 418 gives only the glucose enzyme. (Both strains produce gas while growing on an inorganic medium with glucose.)

(iii) *Bact. dispar.* This organism produces neither a formic nor a glucose hydrogenlyase no matter on what medium the culture is grown. It does not form gas from formate or glucose in Durham tubes. It is therefore entirely unable to decompose formate or glucose with the liberation of hydrogen, either while growing or when in washed suspension.

(iv) *Bact. cloacae.* Two strains of this species were used. The results are summarised in Table IV.

Table IV. *Hydrogenlyases of* Bact. cloacae.

Medium	Conditions	Final p_H	Formic hydrogenlyase: mm.^{3} of H_2 per mg. N	Formic hydrogenlyase: Q_{H_2}	Glucose hydrogenlyase: mm.^{3} of H_2 per mg. N	Glucose hydrogenlyase: Q_{H_2}
Strain 259						
Broth	R.	7·1	0	0	0	0
Broth formate	F.N.A.	—	0	0	0	0
,,	R.	7·4	0	0	0	0
Broth glucose	F.N.A.	5·5	3450	410	Very small initial	Very small initial
,,	R.	7·1	2900	335	170	20
,,	F.A.	—	Very small	Very small	0	0
Inorg. glucose	R.	6·0	0	0	0	0
,,	F.N.A.	—	0	0	0	0
Strain 402						
Broth	R.	7·1	0	0	0	0
Broth formate	F.N.A.	7·0	0	0	0	0
,,	R.	7·4	0	0	0	0
Broth glucose	F.N.A.	5·5	1560	200	1610	190
,,	R.	7·4	3100	370	200	24
,,	F.A.	7·3	170	20	395	47
Inorg. glucose	R.	—	0	0	0	0
,,	F.N.A.	—	0	0	0	0

R. = Roux. F.A. = Flask, aerated. F.N.A. = Flask, not aerated.

Broth alone or broth formate give no hydrogenlyases in whatever conditions growth takes place. The only medium which results in the formation of the enzymes is broth glucose. The formic hydrogenlyase of strain 259 is best formed in the presence of little air. Strong aerobiosis almost completely inhibits its formation. The glucose enzyme on the other hand is most developed in moderately aerobic conditions. In the case of strain 402, the converse occurs. Here it is the formic enzyme which is at its maximum in

moderately aerobic conditions, and the glucose enzyme which is best formed when little air is present.

No enzymes are formed on an inorganic medium, although, as with *Bact. coli* and *Bact. lactis aerogenes*, gas is formed during growth on such a medium containing glucose.

(v) *Bact. freundii.* This is an organism of the coli-typhosus group. It was first described by Braak [1928]. Its chief biochemical interest lies in the fact that it is able to grow anaerobically in an inorganic medium with glycerol as sole carbon source. This is attributed to its power of oxidising one molecule of glycerol at the expense of a second, which is simultaneously reduced to trimethyleneglycol.

Table V gives the results of a number of experiments undertaken to discover what media are necessary for the production of the hydrogenlyases by this organism. In all cases except those specifically mentioned, growth was allowed to take place for 15–18 hours in Roux bottles.

Table V. *Hydrogenlyases of* Bact. freundii.

Exp. No.	Medium	Final p_H	Formic hydrogenlyase: mm.³ of H_2 per mg. N	Formic hydrogenlyase: Q_{H_2}	Glycerol hydrogenlyase: mm.³ of H_2 per mg. N	Glycerol hydrogenlyase: Q_{H_2}
1	Broth	7·3	1710	210	330	40
2	,,	7·3	2100	260	600	75
3	Broth formate	7·8	3500	430	—	—
4	,,	8·0	7400	920	690	85
5	,,	—	4300	535	290	36
6	Broth formate glycerol	7·3	3020	375	900	110
7	,,	7·4	2700	335	750	93
8	,,	7·3	3600	445	1350	170
9	,,	7·4	2900	360	1020	125
10	,,	7·3	—	—	1600	200
11	,,	—	2450	305	1000	125
12	Broth glucose	—	410	50	(240	30)
13	Inorg. glycerol (aer.)	—	0	0	0	0
14	,, (anaer.)	—	0	0	0	0
15	,, chalk (aer.)	—	0	0	0	0
16	,, ,, (anaer.)	—	0	0	0	0
17	Inorg. glucose (aer.)	—	0	0	0	0
18	,, chalk (aer.)	—	340	42	(340	42)
19	Inorg. acetate	—	0	0	0	0
*20	Inorg. broth glycerol	6·8	230	28	140	17
21	,,	—	4000	495	2000	250
22	Inorg. gelatin glycerol	—	0	0	0	0
23	Inorg. albumin glycerol	—	+	+	+	+
24	Inorg. amino-acids glycerol	—	0	0	0	0
25	Broth nitrate	—	0	0	0	0
26	Broth nitrate formate	6·8	240	30	0	0

* Exp. 20 was made with diluted broth.

Exps. 13–18 were all repeated several times. The only positive result—that of Exp. 18—was obtained by growing the culture for 40 hours. An 18-hour culture possessed no lyases.

In Exps. 12 and 18 glucose hydrogenlyase was investigated instead of the glycerol hydrogenlyase.

The media, broth and inorganic, were always diluted 1:3 from stock media. The medium in Exp. 20 contained 10 % of the undiluted broth and 33 % of the undiluted inorganic medium. That in Exp. 21 contained 33 % undiluted broth and 33 % undiluted inorganic medium; that is, it contained the normal amount of broth, as used in Exps. 1–12.

It will be seen that the values of the Q_{H_2} for bacteria grown on the same medium, and acting on the same substrate vary considerably. However, the ratio of the values for the different substrates acted upon by bacteria grown on the same medium is fairly constant. The ratio of the rates of action on glycerol and on formate has also been found in a number of cases where the Q_{H_2} is not known, the nitrogen value of the suspension not having been determined.

Bact. freundii grown on broth alone aerobically acts on glycerol at about one-quarter of the rate at which it acts on formate. Three determinations (Exps. 1 and 2, and another in which the nitrogen was not estimated) give 19, 28 and 25 %—average 24 %. Grown on broth anaerobically, the ratio is 15 %. When formate is present, the rate for glycerol remains about the same as on broth alone aerobically, whilst the rate for formate is approximately doubled. The ratios Q_{H_2} for glycerol/Q_{H_2} for formate in four experiments are 9, 7, 6 and 10 %—average 8 %. Grown on broth glycerol, suspensions give the ratios 63, 67 and 72 %—average 67 %. If both glycerol and formate are present, the ratio is 34 %.

We see then that the addition of formate to the medium increases the amount of formic hydrogenlyase produced, whilst the addition of glycerol increases both. A mixture of formate and glycerol gives a proportion of glycerol hydrogenlyase intermediate between those obtained in the presence of formate or glycerol alone.

Considering the results in Exps. 1–12 alone, one would have to say that, according to Karström's definition of constitutive and adaptive enzymes, the hydrogenlyases of both formic acid and glycerol are constitutive, since they are found when the organism is grown in the absence of these substrates. There are, it is true, small amounts of formic acid produced in the broth, and a similar suggestion may be made with regard to the presence of the hydrogenlyases in *Bact. freundii* when grown in broth alone as was made in the case of *Bact. coli* grown anaerobically on broth. One would then assume that *Bact. freundii* is more sensitive to the presence of formic acid than is *Bact. coli*.

The results of Exps. 13–24 show that only in very special conditions are the enzymes formed on a medium which does not contain broth. When the organism is grown on an inorganic medium, the enzymes are almost invariably absent. Small amounts of the formate and glucose hydrogenlyases are formed when the cells are grown in the presence of chalk, on a medium containing glucose. On a medium containing glycerol, aerobically or anaerobically, in the presence or absence of chalk, the hydrogenlyases are never produced. The non-formation of the enzymes when the culture is made in an inorganic

medium is not due to an inhibitory substance present in this. Exp. 21 shows that if the normal amount of broth is present, the amount of enzyme produced is the same as if no inorganic medium is present. If less broth is present (Exp. 20), a proportionately smaller amount of hydrogenlyase is formed.

It was thought that the absence of the hydrogenlyases when *Bact. freundii* is grown on an inorganic medium might be due to a destruction of the enzymes by the acid formed from the glycerol. However, when a large amount of phosphate buffer was added to the inorganic glycerol medium, no enzymes were formed. This was not due to a poisoning action of the buffer, since a similar amount of buffer added to broth medium resulted in no reduction of the hydrogenlyase activity.

Bact. freundii produces gas whilst growing on an inorganic medium + glucose.

Some experiments were performed in order to investigate the effect of formate in an inorganic medium. It was found, however, that the presence of formate entirely inhibited growth in several media in which growth otherwise occurs.

Unhydrolysed protein is not sufficient to ensure the production of any appreciable amount of enzyme (Exps. 22, 23). 0·5 % gelatin in the medium gives no hydrogenlyases at all. The presence of 0·5 % albumin gives a very small amount of enzyme. The Q_{H_2} was not measured since some of the protein was precipitated and consequently the nitrogen value did not give a true measure of the amount of bacteria. However, a very thick suspension was used, and the rate of hydrogen output was only just measurable.

A mixture of amino-acids was then tried. The medium contained about 1 % of a mixture of glycine, alanine, leucine, tyrosine, tryptophan, histidine, glutamic acid and aspartic acid, together with inorganic medium and 0·5 % glycerol. The suspension of bacteria which had been grown on this medium did not possess the hydrogenlyases.

It will be seen from Table V that nitrate entirely inhibits the production of the enzymes from broth alone. The addition of nitrate to a broth formate medium results in the formation of a small amount of the formic hydrogenlyase but of no glycerol hydrogenlyase.

Discussion.

Hydrogenlyases are formed by many species of bacteria in certain conditions. The factors influencing their formation are seen to vary somewhat with the organism studied, but the following general conclusions may be drawn.

Aeration has an inhibitory effect on the formation of the hydrogenlyases. This is not due to a destruction of the enzymes. Once the enzymes have been formed, aeration of a washed suspension of the bacteria, in which the enzymes are present, does not destroy them.

Suspensions of the two strains of *Bact. lactis aerogenes*, and *Bact. cloacae*, strain 259, were aerated for 24 hours at room temperature. For the same

period, portions of the same preparations were left in a vacuum, also at room temperature. The activities of the hydrogenlyases were determined before and after the experiment (Table VI).

Table VI. *Effect of aeration on activity of hydrogenlyases.*

	Q_{H_2} (formic hydrogenlyase)			Q_{H_2} (glucose hydrogenlyase)		
Bacterium	Initial	After vacuum 24 hrs.	After aeration 24 hrs.	Initial	After vacuum 24 hrs.	After aeration 24 hrs.
Bact. lactis aerogenes						
Strain 124	225	225	200	35	100	73
Strain 418	250	250	270	35	130	50
Bact. cloacae						
Strain 259	510	435	375	75	175	75

The formic hydrogenlyases are at most slightly decreased either by keeping in a vacuum or by aeration. The effect on the glucose enzyme of keeping in a vacuum is to increase it considerably. Aeration, on the other hand, results in rather less increase.

It may be concluded, therefore, that aeration of a growing culture does not result in the destruction of formed enzymes but actually prevents their formation. This may possibly be due to the oxidation of some substance in the medium necessary for the production of the enzymes.

A second factor is the presence of broth. Although all the organisms except *Bact. dispar* form hydrogen from glucose while growing in an inorganic medium, only *Bact. lactis aerogenes* and, in very special conditions, *Bact. freundii* give the enzymes in washed suspension of cultures from this medium. It is possible that the enzymes in these conditions are less stable, or formed in extremely small amounts, and are thus found only in some cases. The problem is being further investigated.

A third factor is the presence of the substrate. The influence of the substrate on the production of organisms possessing the hydrogenlyases may be pictured as either (1) a natural selection, or (2) a chemical adaptation. In the former case, one must suppose that there exists in all cultures a small but definite number of cells possessing the enzyme. Since at some period each of the organisms used has been grown from a single cell colony, one must imagine a biochemical variation or mutation of a definite though low frequency, in order to account for the existence of the cells containing the hydrogenlyases. Such variations have been known to occur. Neisser [1906] described the sudden appearance in a certain coliform bacterium of an ability to ferment lactose, and Massini [1907] showed that the new strain was immunologically identical with the old strain. Enzymes, then, arising by some such mutation, become, according to this hypothesis of natural selection, of biological value to the organism. Those members possessing them are therefore at an advantage and tend to multiply at the expense of the others. A strain is thus formed in which the majority of the members possess the enzyme in question.

Such an explanation presupposes that the reaction catalysed by the enzyme is of service to the organism. In the case of the formic hydrogenlyase this is difficult to demonstrate. The reaction $HCOOH = H_2 + CO_2$ can hardly be of any value to the cell. The heats of reaction show that it is only slightly exothermic, and although the data for the free energy change are not known, it is probable that little or no energy is available to the cell. Moreover, the end products cannot enter into the cell structure. It is of course possible that formate is detrimental to the organism and that the action of the hydrogenlyase permits of its removal anaerobically. Such an explanation is supported by the following facts: (1) the hydrogenlyases are not formed in highly aerobic conditions, where the removal of formate occurs by the action of formic dehydrogenase, and (2) in inorganic media where growth normally takes place, sodium formate is entirely inhibitory (see above *Bact. freundii*).

On the other hand, it is difficult to see how this applies to the cases of glucose and glycerol.

Against the selection hypothesis the following experiment may be quoted. *Bact. freundii* was grown for a month, with frequent sub-culturing, on broth glycerol in one case, and on broth formate in another. After this time, an inoculation was made from each of these into plain broth, and the Q_{H_2} values for formate and glycerol determined in each case after the usual 15 hours' growth. It was found that these were of exactly the same order as was obtained when the inoculation was made into plain broth from "untrained" *Bact. freundii*.

Hence, if selection had occurred during cultivation on the glycerol or formate medium, the character acquired was immediately lost on returning to plain broth. This must again involve natural selection of the few remaining unaltered cells. This presupposes that the loss of the hydrogenlyases is an advantage when the cells are growing in plain broth—a supposition difficult to support.

More plausible is the second suggestion, that of chemical adaptation. On this view, an adaptive enzyme arises as a response to its chemical environment. It would then partake of the nature of an acquired character in higher organisms. With the removal of the stimulus, the character is lost by the descendants of the organism. They still, of course, retain the power to develop that character in conditions similar to those in which the parent organism developed it. This explanation, though admittedly incomplete, is in accordance with the experimental facts so far determined.

In this way, one would distinguish between "training" and "adaptation" by considering the former to be an inheritable variation, whilst the latter is a specific response to a change in the environment and is hence of the nature of an uninheritable acquired character.

SUMMARY.

1. The conditions governing the formation of the hydrogenlyases in several species of bacteria have been studied.

2. A method for measuring the activity of the culture has been worked out. The unit employed is the Q_{H_2}, *i.e.* mm.^{3} of hydrogen liberated per mg. of dry bacteria per hour.

3. Aerobic conditions operating during growth prevent the formation of the enzymes in some cases and greatly decrease their production in others.

4. Aerating a washed suspension does not destroy the enzyme. It is therefore the actual formation of the enzyme which is inhibited by air.

5. In most cases, no hydrogenlyases can be demonstrated in organisms grown on a simple inorganic medium, whereas organisms grown in presence of broth contain them.

6. The presence in the growing culture of the substrates on which the enzymes act is either a necessary factor in their production or very greatly increases the amount formed.

7. The formation of a culture which possesses the hydrogenlyases is probably the result of a specific action on the growing organisms of certain substances in the medium in which they are grown. It is unlikely that a natural selection of pre-existing enzyme-containing organisms occurs.

8. A distinction is drawn between "training" and "adaptation." The former seems to be a selection of a variation, the latter a direct response to the stimulus of the chemical environment.

I am glad to take this opportunity of expressing my gratitude to Miss Marjory Stephenson at whose suggestion this work was undertaken and whose advice and constant encouragement were invaluable. My thanks are also due to Sir F. G. Hopkins for his interest in this work.

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CCXXII. PYRUVIC ACID AND VITAMIN B_1 DEFICIENCY.

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The significance of pyruvic acid in carbohydrate metabolism of animal tissues has long been discussed. Since Meyerhof, Lohmann and Meier [1925] showed that out of a large number of substances investigated only pyruvic acid and lactic acid increased the oxygen uptake and carbohydrate content of muscle, pyruvic acid has been suggested as a stage in the oxidation or oxidative resynthesis of lactic acid. Only recently, however, have Case and Cook [1931] been able to detect pyruvic acid in muscle in the absence of fixative under both aerobic and anaerobic conditions. A discussion of the possible origin and fate of this pyruvic acid is given in a recent paper by Case [1932, 2].

We have shown [Gavrilescu and the authors, 1932] that the action of vitamin B_1 concentrates *in vitro* was specifically related to the lactic oxidase system of pigeon's brain; no evidence could be obtained of interaction with the succinoxidase system. A desire to settle the point of action of the vitamin concentrates led us to the study of the behaviour of normal and avitaminous pigeons' brains in the presence of pyruvic acid. The experiments were made more interesting by the realisation that light might be thrown upon the fundamental problem of the rôle of pyruvate in carbohydrate metabolism.

Our work has elicited the unexpected fact that the oxygen uptake in the presence of pyruvate is always much more depressed than with lactate in the avitaminous brain; further, contrary to experience with lactate, this depression is not correspondingly improved by the addition of vitamin B_1 concentrates *in vitro*. Normal brains show good oxygen uptakes with pyruvate as substrate. We have not been able to establish a satisfactory explanation of these facts.

Experimental.

Method. The oxygen uptake of minced pigeon's brain has been determined as previously described [Gavrilescu *et al.*, 1932]. The estimations have all been done at 38° with 50–100 mg. of minced tissue suspended in 3·0 cc. of phosphate buffer solution. The vitamin B_1 concentrate had an activity of approximately 0·05 mg. for the bird and was the same as previously used. Vitamin B_1 deficiency was induced in the birds by our usual methods.

Pyruvic acid. The literature dealing with pyruvic acid in relation to oxidase and other biological systems is singularly silent upon the methods used to ensure the purity of the acid under experiment[1]. For our purposes freedom from the last traces of lactic acid, and of possible toxic impurities (see below) was important. We have therefore relied upon one or two redistillations of a pure commercial sample (Messrs Boots and Co.) and have in many cases used freshly distilled acid. Our samples did not crystallise, but distilled within a close range of 0·5–1·0° at pressures of 15–20 mm. Hg and were quite colourless. After keeping for 1 month the titration of one sample gave 93 % —COOH and 88 % by iodine value. Specimens of the 2:4-dinitrophenylhydrazone prepared direct from aqueous solutions gave the correct melting-point, and we have little doubt that a sufficient approach to purity was made for our purposes.

Results.

Oxygen uptake in the presence of varying concentrations of pyruvate.

Table I shows the oxygen uptake of normal and avitaminous cerebrums and optic lobes/rest in the presence of varying concentrations of pyruvate. Fig. 1 shows typical curves for normal and avitaminous cerebrums. The

Table I. *Effect of varying concentrations of pyruvate.*

Oxygen uptake in mm.³/g./hr.

Exp.	Ringer	Lactate	Pyruvate 0·007 *M*	Pyruvate 0·017 *M*	Pyruvate 0·034 *M*	Pyruvate 0·102 *M*
			Normal brains.			
			(*a*) Cerebrums.			
264	(850)	—	1750	1930	1705	(1540)
276	770	2150	2040	2120	2230	2000
278	740	1960	1980	(2350)	2300	(2050)
279	1005	1860	1710	1680	1935	1575
			(*b*) Optic lobes/rest.			
264	510	—	—	—	(1300)	1200
286	635	—	1830	2005	—	—
			Avitaminous brains.			
			(*a*) Cerebrums.			
262	775	—	1085	970	925	735
265	740	—	1010	(1080)	975	895
			(*b*) Optic lobes/rest.			
265	(660)	—	875	(870)	790	—

apparent Michaelis constant for the enzyme systems under these conditions is of the order of 0·003 *M*, both for normal and avitaminous brains. This is approximately the same as the constant previously obtained for lactate under similar conditions, if we allow for the presence of inactive lactate in the racemic mixture.

[1] Since the completion of this work, the papers by Case [1932, 1, 2] have appeared. The paper by Wendel [1932] also appeared while this was in progress.

A factor, however, appears which was not present in our previous experiments with succinate and lactate, namely inhibition with higher concentrations. This effect was obtained with our most highly purified specimens, and in our opinion cannot be due to a toxic impurity. Bernheim [1928] showed that pyruvic acid in the same concentrations had an inhibitory effect on the lactic acid dehydrases of yeast. We have found no evidence of pyruvic acid inhibiting the oxygen uptake in the presence of lactate at concentrations below 0·034 *M*. As a result of this inhibitory effect there is no broad optimum for maximum oxygen uptakes. In one experiment the maximum was at 0·007 *M*, and the inhibition started at 0·017 *M*. In another there was no inhibition with 0·03 *M* pyruvic acid. We have worked with 0·007 *M* and 0·034 *M* pyruvate, and although we cannot be certain that these will give maximum uptakes, they will not be far from the optimum.

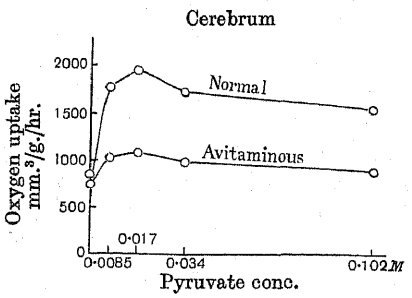


Fig. 1.

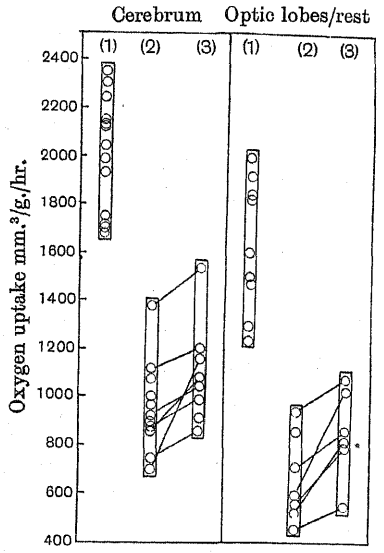


Fig. 2.

Fig. 1. Oxygen uptake of cerebrum from pigeon, normal and avitaminous, in relation to concentration of pyruvate.

Fig. 2. Oxygen uptake of pigeon's brains, (1) normal, (2) avitaminous, (3) avitaminous + vitamin B_1 concentrate.

Oxygen uptake for normal and avitaminous brains.

(1) *Comparisons with lactate and pyruvate as substrates.* Fig. 2 shows all the results obtained with normals using pyruvate as substrate. Most of the figures are in Table I and the averages are in Table II. Table III contains all the results for avitaminous brains and the effects of addition of vitamin B_1 concentrates *in vitro*.

It will be seen that the normal brains take up as much oxygen in the presence of pyruvic acid as in the presence of lactic: indeed the averages are

Table II. *Pyruvate results.*

Normals.

Oxygen uptake in mm.³/g./hr.

	Lactate	Pyruvate 0·007 *M*	Pyruvate 0·017 *M*	Pyruvate 0·034 *M*
Average cerebrum	1620	1870	2010	1895
Average optic lobes/rest	1380	1840	1800	1480

Table III.

Oxygen uptake in mm.³/g./hr.

Exp.	Lactate	Pyruvate Concentration *M*	Pyruvate Control	Pyruvate Vitamin	Increase %
		Avitaminous birds.			
		(*a*) Cerebrums.			
238	—	0·034	730	870	19
241	(1360)	0·034	900	995	10
245	1160	0·034	705	(1165)	66
249	1375	0·034	890	915	2
266	—	0·034	900	1050	17
300	1430	0·017	1380	(1550)	12
301	1290	0·017	(1340)	1600	19
266	—	0·017	885	1090	23
262	—	0·017	970	—	—
265	—	0·017	(1080)	—	—
267	1200	0·007	920	1210	30
281	(1150)	0·007	(1125)	1210	8
262	—	0·007	1085	—	—
265	—	0·007	1010	—	—
		Average	990	1160	20
		(*b*) Optic lobes/rest.			
245	—	0·034	600	(1040)	70
248	—	0·034	460	560	22
249	—	0·034	570	810	42
262	—	0·034	530	830	57
300	1130	0·017	(955)	8090	14
301	(980)	0·017	770	1140	48
280	—	0·007	720	875	21
265	—	0·007	875	—	—
		Average	610	910	40

higher. On the other hand the uptake of the avitaminous brains is very much lower than for lactate, *e.g.* Exp. 245; residual oxygen uptake 665; pyruvate 705; lactate 1160. It might be considered from these results that depressed oxidation with pyruvate represents a more fundamental lesion than that with lactate. The effect of addition of vitamin B_1 concentrates, however, does not confirm this.

(2) *Addition of vitamin B_1 concentrates.* Reference to Table III and Fig. 2 shows that the effect of the concentrate is very variable. Often the increase can be entirely explained by the effect on the residual uptake. Often, especially in the optic lobes/rest, the effects are large, but in no case does the concentrate ever raise the uptake above 1140 mm.³/g./hr., where the minimum normal

figure is 1550, and the average 1850. The vitamin acting *in vitro* is unable to restore the oxidising powers to anything approaching normal with pyruvate as substrate. This suggests that the change is not connected with the deficiency of vitamin B_1 directly. If this interpretation of the results is correct, it raises two possibilities. The failure of the avitaminous brain to take up oxygen in the presence of pyruvic acid might be due either to changes in the brain tissue secondary to the vitamin B_1 deficiency, or to the absence from the avitaminous minced brain of some necessary somatic factor (supplied by the blood stream).

Birds killed with iodoacetic acid.

With regard to the first possibility, the only change of which we know, apart from changes in oxidation, is the increase in lactate in avitaminous as compared with normal brains. If lactate and pyruvate were both oxidised at some common centre, it might be that the increased concentration of lactate blocked the pyruvate. This we have tried to test by killing birds with iodoacetate (IAA), which is known to inhibit lactic acid production in these brains. Kinnersley and Peters [1930] for instance found in normals killed with IAA 20–30 mg./100 g. of lactic acid and in avitaminous 30–60 mg.[1] This would be quite different from the normal 100–150 mg./100 g. and avitaminous 150–250 mg./100 g. The dose of IAA used for killing the birds was 75 mg./1000 g. body weight. For safety we have used IAA in the bottles during the experiments in a concentration 0·1 mg. per 1 cc. of Ringer as suggested by Fisher [1931]. This has some effect on oxidation both with pyruvic acid and lactic acid, as the poison is not absolutely defined in its action [Lohmann, 1931], and with increased concentrations glyoxylase systems are affected [Dudley, 1931]. The decrease is shown in Table IV. With normal brains the pyruvate and lactate

Table IV. *Iodoacetic acid experiments.*

Oxygen uptake in mm.^{3}/g./hr.

	Cerebrums			Optic lobes/rest		
Exp.	Ringer	Lactate	Pyruvate	Ringer	Lactate	Pyruvate
			(*a*) *Normal brains.*			
289	460	(1150)	1135	380	1125	(1120)
293	(440)	(1380)	1300	385	945	1100
296	550	1610	1165	515	1405	1095
299	500	1390	1240	(425)	1060	1070
			(*b*) *Avitaminous brains.*			
288	490	(1030)	(775)	—	560	(510)
294	505	970	(670)	—	650	450
297	530	1160	900	320	780	550
298	430	1000	670	390	750	580

oxygen uptakes are almost identical. The extra oxygen uptake for pyruvate has disappeared. In one experiment, No. 296, however, the pyruvate oxidations are much more markedly depressed than the lactate. There is an indication that the IAA has a possible specific inhibitory effect on pyruvic acid

[1] The cerebrum generally gave the lower figures.

oxidations, and this must moderate any conclusions drawn from these experiments. In the avitaminous brains there is still the increased depression of oxygen uptake in the presence of pyruvate and there is no marked improvement on vitamin addition. Even with the lowered lactic acid content of the IAA brains the pyruvate is less efficient. Hence there is no indication that the changes in pyruvate behaviour are due to blockage by lactate.

The oxygen uptake in the presence of pyruvic acid of the brains of birds cured by dosing with concentrates.

The authors [1932] had previously investigated the oxygen uptake of cured avitaminous birds' brains in the presence of lactate. Table V gives figures for pyruvate and lactate for two groups of birds both dosed with 8–12 pigeon

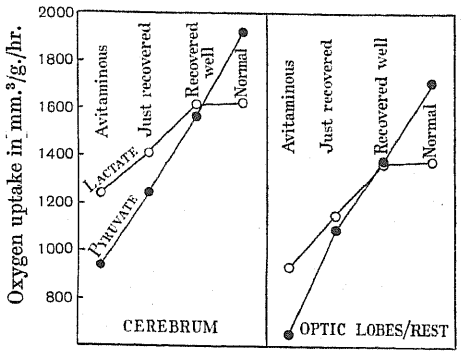


Fig. 3. Chart showing oxygen uptake (average) in relation to condition of bird, avitaminous, just recovered, recovered well, normal. ● pyruvate, ○ lactate as substrate.

Table V. *Dosed birds.*

Oxygen uptake in mm.³/g./hr.

	Cerebrums				Optic lobes/rest	
	Lactate	Pyruvate			Lactate	Pyruvate
Exp.		Concentration *M*	Control	Vitamin		
		(*a*) *Birds just out of symptoms.*				
291	1430	0·017	(1200)	(1235)	995	890
290	1325	0·017	1280	(1290)	1395	1085
274	1350	0·007	1320	1790	1225	1320
284	1380	0·017	(1220)	(1270)	1270	1040
273	1520	0·007	(1355)	1600	(1220)	1100
285	(1430)	0·017	(1100)	1510	1005	1005
Average	1410		1245	1450	1140	1070
		(*b*) *Birds well recovered.*				
283	1665	0·017	1580	(1770)	(1380)	(1410)
292	1540	0·017	1500	—	(1295)	(1285)
272	(1480)	0·007	1405	(1570)	(1350)	—
277	1685	0·007	(1730)	1720	1420	—
282	1660	0·017	1780	1600	1380	(1475)
275	1650	0·007	(1400)	1790	1405	1270
Average	1615		1565	1690	1370	1380

doses (approximately vitamin B_1 units). Group (*a*) had only just recovered from head retraction and were very weak. Group (*b*) had had a longer time for recovery. The averages for lactate and pyruvate for each group and for normals and avitaminous are shown in Fig. 3. There is a recovery of oxygen uptake in the presence of pyruvate, but it is not so complete as for lactate. No relationship has been found between the degree of recovery with the two substrates in individual birds. We feel that these experiments indicate that the fundamental lesion associated with the nervous symptoms is concerned with the lactate, and that the change in behaviour to pyruvate is in some way secondary.

Discussion.

These experiments make it difficult to believe that pyruvic acid is a normal stage in oxidation of lactic acid, unless it be assumed, as suggested by Neuberg [1927] that pyruvic acid formed in the tissues molecule by molecule is constitutionally different from added pyruvic acid. In this respect, we are in agreement with Case and Cook [1931] and Case [1932, 1, 2].

We have thought that for the normal oxygen uptake of pyruvate to be regained by avitaminous tissue a somatic factor might be required, whose co-operation with the vitamin is needed for normal oxidations in the presence of pyruvic acid. We cannot say whether such a factor is needed to remove a toxic substance in the tissues or to provide a co-ferment. A few experiments with addition of serum have given very varied and inconclusive results.

Summary.

1. Normal pigeon's brain when minced gives a large oxygen uptake in the presence of pyruvic acid.

2. The avitaminous pigeon's brain gives a very low oxygen uptake in the presence of pyruvic acid, which is not restored by addition of vitamin B_1 concentrate *in vitro*. These same concentrates are capable of restoring lowered oxygen uptake with lactate as substrate.

3. The defect in oxidation is not associated with the high lactic acid content of the avitaminous brains.

4. There is no indication that pyruvic acid is a stage in the oxidation of lactic acid in the pigeon's brain.

We are indebted to Mr H. W. Kinnersley for the preparation of the concentrates used in these experiments, also to the Government Grant Committee of the Royal Society and to the Medical Research Council for grants towards the cost of the experiments.

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CCXXIII. THE INFLUENCE OF THE ADMINISTRATION OF ARGININE AND HISTIDINE ON THE EXCRETION OF CREATININE.

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(*Received September 28th, 1932.*)

The numerous attempts to demonstrate the origin of muscle-creatine or urinary creatinine from arginine or histidine have been almost entirely negative in result or open to serious criticism. The fact that both arginine and histidine are subject to destruction by the liver has no doubt militated against any positive result in the majority of cases, especially when small amounts of the amino-acids have been administered *per os*.

In regard to histidine more definite evidence has recently been presented by Abderhalden and Buadze [1929; 1930], who have shown that feeding large amounts of histidine or haemoglobin led to significant increases in creatinine excretion.

Reviews of the literature dealing with the effect of administering arginine, histidine, cystine and other substances are given by Hunter [1928], Hyde and Rose [1929] and Abderhalden and Buadze [1929]. In a recent paper Beard and Barnes [1931] reported the effects of administration of protein, amino-acids and related substances on muscle-creatine and urinary creatinine in adult rats and also the effects of these substances on creatinine elimination in man. A large number of amino-acids, including histidine, arginine, glycine, glutamic acid, tyrosine and cystine, the proteins edestin and caseinogen, and finally choline and glycocyamine were found to increase muscle-creatine. Negative results were obtained with sarcosine, nucleic acid, betaine and taurine. All the amino-acids and proteins studied increased the elimination of creatinine.

These authors believe that their results cannot be ascribed to the effect of specific dynamic action and conclude that the creatine formation and creatinine excretion were due either to feeding large quantities of proteins and amino-acids or to the possibility that these substances stimulated creatine-creatinine metabolism in some other way than by specific dynamic action. They favour the former view but taking into account the large number and variety of substances giving positive results a more likely explanation is one based on the assumption of a generalised stimulation of creatine-creatinine metabolism. However, several investigators [Lewis, Dunn and Doisy, 1918;

Zwarenstein, 1928; Christman and Mosier, 1929] have shown that administration of several amino-acids, including glycine which gave the maximum effect in Beard and Barnes' experiments, had no effect on creatinine excretion. A satisfactory explanation of the above results is difficult and the findings of Beard and Barnes are in obvious need of re-examination.

In the work described below attention has been focused largely on the effects of arginine and histidine but some of the experiments of Beard and Barnes with other amino-acids have been repeated. During the course of research on other lines in this laboratory it was found that rabbits were very suitable animals for investigations on creatine-creatinine metabolism. The daily output of creatinine is constant within comparatively narrow limits except for slight seasonal variations and is furthermore independent of caging or of the usual diet of carrots, cabbages and bran. For short period experiments such as those described below the normal daily creatinine excretion remains remarkably constant.

Experimental.

The experiments were carried out on adult male rabbits. Details in regard to caging, feeding and collection of urine have been described elsewhere [Schrire and Zwarenstein, 1932]. Creatine and creatinine were estimated by Folin's method. Eight animals were used in all and before the effects of the various substances were investigated the daily creatinine excretion was estimated for periods of 4 or 5 days over several weeks and thus a normal preperiod consisting of about 18 daily creatinine figures was obtained. On the day preceding administration of the substance creatinine was again determined thus checking any large deviation from the normal.

The histidine hydrochloride, arginine, glycine and alanine were dissolved in Locke's solution (30–35 cc.) and the solutions of the first two were neutralised with dilute NaOH or HCl before injection. Cystine, tyrosine, glutamic acid and glycocyamine were fed mixed with bran. Only in the case of cystine did the animals fail to ingest all the mixture, so that the amounts for cystine in Table II are actually rather less than stated. In some experiments the animals received a single injection, in the majority of experiments they were injected or fed once a day on three consecutive days. Four animals were usually put into separate metabolism cages at the same time. One of these always received Locke's solution only or bran and served as a control. Every animal in its turn served as a normal control in the various experiments. In all cases the diet consisted of cabbages and carrots. The controls for the feeding experiments were given bran in addition. For the sake of convenience the data in the following Tables are arranged in a slightly different sequence from that in which they were actually obtained. Injection of 35 cc. of Locke's solution daily for 3 days had no effect on the excretion of creatinine. Similarly, the addition of bran to the usual ration had no effect. Small quantities (10 to 50 mg.) of the different amino-acids were added to 10 cc. portions of a filtered

urine, and the creatinine was estimated and compared with the value obtained from the same urine without the addition of the amino-acid. The readings were identical in all cases except on addition of alanine or cystine and for these two substances the differences were not significant.

The figures in the Tables below unless otherwise stated refer to mg. creatinine excreted in 24 hours. The figures in brackets refer to the amount of the substance in g. fed or injected.

Table I.

Rabbit No. ...	I	II	III	IV
Weight (kg.) ...	2·46	2·52	2·18	2·03
A. *Pre-period*				
No. of 24 hour periods	18	16	18	17
	96 ± 2	98 ± 5	90 ± 6	92 ± 5
B. *Arginine*				
Preceding day	100	101	90	95
Injection period (1 day)	109 (5)	136 (5)	106 (5)	89 (2·5)
	103	101	103	99
	107	93	93	103
	102	110	80	99
Preceding day	—	—	—	91
Injection periods (3 days)	—	—	—	91 (2·5)
	—	—	—	114 (3·0)
	—	—	—	114 (5·0)
C. *Histidine*				
Preceding day	99	106	84	92
Injection period (1 day)	94 (3)	138 (5)	104 (3·5)	109 (3·5)
	100	126	96	96
	110	109	85	80
	103	113	—	—
Preceding day	104	—	93	—
Injection periods (3 days)	125 (2·5)	—	93 (2·5)	—
	96 (3·5)	—	96 (3·0)	—
	117 (4·0)	—	120 (5·0)	—

Table II.

Rabbit No. ...	III	IV	V	VI
Weight (kg.) ...	2·18	2·03	1·80	1·77
A. *Pre-period*				
No. of 24 hour periods	18	18	18	18
	90 ± 6	92 ± 5	72 ± 4	72 ± 3
B. *Glycine*				
Preceding day	92	90	75	70
Injection periods (3 days)	91 (4·5)	85 (4·5)	65 (5)	70 (5)
	96 (3·5)	96 (3·5)	78 (5)	73 (5)
	91 (3·5)	92 (3·5)	70 (3)	69 (3)
C. *Alanine*				
Preceding day	90	93	72	80
Injection periods (3 days)	92 (4·5)	87 (4·5)	67 (4)	70 (4)
	80 (3·5)	93 (3·5)	72 (4)	75 (4)
	90 (3·5)	80 (3·5)	65 (3)	72 (3)
D. *Cystine*				
Preceding day	82	90	—	80
Feeding periods (2–3 days)	83 (5)	90 (3·5)	—	70 (3·5)
	80 (1)	90 (4)	—	69 (4)
	86 (4)	—	—	65 (3·5)

Table III.

Rabbit No. ...	III	IV	VII	VIII
Weight (kg.) ...	2·18	2·03	1·80	1·99
A. *Pre-period*				
No. of 24 hour periods	18	18	18	18
	90 ±6	92 ±5	73 ±5	76 ±6
B. *Tyrosine*				
Preceding day	85	91	71	73
Feeding periods (2–3 days)	90 (5)	89 (4·5)	76 (5)	70 (5)
	93 (5)	94 (10)	70 (5)	75 (5)
	85 (3)	—	73 (3)	80 (3)
Glutamic acid				
Preceding day	96	92	69	73
Feeding periods (2–3 days)	90 (5·5)	89 (7)	70 (5·5)	73 (7·0)
	98 (3·0)	93 (5·5)	69 (3·0)	77 (5·5)
	85 (4·0)	87	69 (4·0)	76

Glycocyamine

Rabbit No.	II		III	
	Preformed creatinine	Creatine	Preformed creatinine	Creatine
Preceding day	80	0	84	0
Feeding periods (2–3 days)	92	39 (3)	91	179 (3)
	95	47 (4)	68	269 (3)
	90	107 (3)	—	—
	94	161	—	—

Variable amounts of creatine (0–36 mg.) were found in the urine after injection of arginine and histidine, but similar amounts were excreted during the normal pre-periods and also after injection of Locke's solution. Consequently the occurrence of creatine after injection of the amino-acids cannot be regarded as significant.

Discussion.

The results in Table I show that the injection of arginine and histidine caused increases in urinary creatinine of the order of 10–40 %. The effects were definite in all the animals injected and although the reaction to arginine and histidine in any one animal was very similar, individual variations occurred. Single day injections into animal II gave increases of about 40 %, in I the increases were less (about 14 %). Injections on successive days caused these increases to persist; in some cases further rises occurred. These effects are in striking contrast to those obtained with other amino-acids. In Tables II and III it is seen that none of the amino-acids, glycine, alanine, cystine, tyrosine and glutamic acid, had any effect on the excretion of creatinine or creatine, whether administered subcutaneously or *per os*. The results in Tables II and III indicate further that the increases obtained with arginine and histidine could not have been due to specific dynamic action.

The large increases in creatine excretion as a result of feeding glycocyamine confirm the results of previous investigators. The preformed creatinine, except for a drop on the second day in animal III, remained unaffected.

It is highly significant that with arginine and histidine which have struc-

tural affinities to creatine and creatinine there are prompt and definite increases in creatinine, while with all the other amino-acids, which have no such direct relationship, there is no effect. Glycocyamine increases creatine excretion but has no effect on creatinine. An analysis of Table I indicates that arginine and histidine are identical in their effects upon urinary creatinine. From this it may be concluded that the iminazole nucleus in histidine and the guanidine nucleus in arginine undergo transformation into creatinine (either directly or through the intermediate stage of creatine).

On the other hand when the guanidine nucleus is introduced in the form of glycocyamine it is transformed directly into creatine which is excreted as such. This difference in effect may be due to the fact that arginine and histidine were injected and glycocyamine was administered *per os*, that is, that the locus of transformation in the former case was muscle and in the latter the liver. Reviewing the evidence in regard to the site of formation of creatinine from creatine Hunter [1928] comes to the conclusion that the liver plays no part in this change and that the muscles are most probably the chief site of creatinine formation. This does not exclude the possibility of creatine formation from glycocyamine in the liver; consequently the liver probably can convert glycocyamine into creatine but cannot convert creatine into creatinine. The creatine is formed from glycocyamine rapidly and in large amounts and is excreted as such. The rapidity with which creatinine arises from arginine and histidine suggests that its formation is a direct one, but the formation of creatine as an intermediary cannot be definitely excluded. The point raised in regard to the different effects obtained with parenteral or oral administration of creatine-creatinine precursors requires further detailed investigation and may lead to an explanation of the contradictory results encountered in the literature.

The above results although not conclusive indicate that arginine and histidine are normal precursors of creatine or creatinine and suggest that only those substances with close direct chemical relationships to creatine or creatinine can be taken into account in any attempt to elucidate the still rather obscure problem of the significant biochemical processes involved in creatine and creatinine metabolism and their final interrelationships.

Summary.

1. Injection of arginine and histidine into adult male rabbits gives rise to a 10–40 % increase in the elimination of urinary creatinine.

2. Feeding with glycocyamine has no effect on creatinine excretion but leads to a large output of creatine.

3. The amino-acids glycine, alanine (injected) and tyrosine, cystine, glutamic acid (ingested) have no effect on creatine or creatinine excretion.

4. It is suggested that the transformation of arginine and histidine into creatinine takes place in the muscles probably *via* creatine and that the glycocyamine-creatine change is a direct one taking place in the liver.

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CCXXIV. THE INFLUENCE OF THE GONADS ON PROTEIN METABOLISM.

II. THE EFFECT OF INJECTIONS OF SALINE SUSPENSIONS OF TESTES AND TESTICULAR EXTRACTS ON URINARY CREATININE AFTER CASTRATION.

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(*Received November 1st, 1932.*)

In a previous paper [Schrire and Zwarenstein, 1932] it was shown that castration of adult male rabbits led to a persistent increase in the excretion of urinary creatinine, that grafting of testes into castrated animals caused a decrease to the normal pre-castration level, and that after subsequent removal of the graft the usual castration effect on urinary creatinine was re-established. These results suggest the existence of an endocrine relationship between the testes and creatinine metabolism, but a definite conclusion can be arrived at only when evidence has been presented that the effects of castration can be compensated by injection of testicular suspensions and extracts. The results in regard to the effect of testicular injection on the increased elimination of creatinine after castration form the substance of this paper.

Experimental.

The procedure with regard to feeding, caging *etc.* was the same as reported in the previous communication. Three to four months after removal of the testes the usual castration effect on creatinine appeared. The testes of rabbits or portions of bull testis were finely divided with a sharp scalpel, the macerated liquid mass suspended in Locke's solution and injected subcutaneously by means of a large veterinary syringe and needle. The effects were controlled by injection of brain suspension prepared in the same way. The testicular extracts were prepared from the testes of bulls, pigs and rams according to the method described by Moore, McGee and co-workers [McGee, Juhn and Domm, 1928; Moore, Price and Gallagher, 1930]. The final extract was taken up in olive oil before injection.

Extracts of sheep's brain prepared by the same method or olive oil alone were injected as controls.

1 cc. of olive oil extract represents 94 g. of fresh testis or 57 g. of brain. In the first part of Table I (pre-period and 3 months castration figures) the

Table I. *Daily urinary creatinine.*

Rabbits Nos. 1, 2, 3. Normal animals.
,, 4 and 5. Castrated 14. iii. 32.
,, 6 and 7. Castrated 16. iii. 32.

Date	Normal (1)	Normal (2)	Normal (3)	Castrated (4)	Castrated (5)	Castrated (6)	Castrated (7)
				mg.			
A. Pre-periods and 3 months castration							
Jan. 17	95	63	63	94	60	74	86
Feb. 1	95	75	78	97	68	81	89
,, 29	97	75	81	94	72	79	93
Mar. 7	94	79	86	87	75	79	87
,, 29	91	—	—	89	—	79	74
Apr. 11	92	—	—	87	—	74	81
,, 25	93	—	—	91	—	79	86
May 16	93	—	—	90	—	93	92
June 15	97	—	—	94	—	84	96
B. Injection of saline suspensions of rabbit's testes							
July 11	79	92	81	116	104	107	100
,, 12	90	85	96	111	91	106	106
,, 13	95	85	79	120	106	114	106
,, 14	98	88	88	112	116*	125*	117
,, 15	92	92	86	103	75	93	108
,, 16	81	85	84	115	62	84	110
,, 17	96	85	82	112	110	105	113

C. Injection of saline suspensions of bull's testis and brain

	(1)	(3)	(5)	(6)
Sept. 9	92	78	116	98
,, 10	86	80	112	104
,, 11	83	74	108	111
,, 12	92	72	112	106
,, 13	92	80†	114†	108†
,, 14	90	92	120	116
,, 15	96	90	85	125
,, 16	100	79	105	121
,, 17	100	Died	124	127
,, 18	100	—	120	127

D. Injection of testicular and brain extracts and olive oil

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
July 23	78	70	85	120	105	105	109
,, 24	80	74	75	109	89	100	109
,, 25	76	80	90	109	104	109	120
,, 26	87	83	92	96	120	83	120
,, 27	93	75	80	96	106	75	109
,, 28	86	75	75	72	120	78	120
,, 29	86	70	51	86	104	100	114
,, 30	85	86	—	98	116	116	127

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
July 25	No injection	Brain 4 cc.	Oil 4 cc.	Testis 4 cc.	No injection	Testis 4 cc.	Brain 4 cc.
,, 26	,,	,,	,,	Testis 6 cc.	,,	Testis 6 cc.	,,
,, 27	,,	,,	,,	Testis 10 cc.	,,	Testis 10 cc.	,,
,, 28	,,	Oil 4 cc.	Testis 10 cc.	Testis 10 cc.	,,	Testis 10 cc.	Oil 4 cc.

* On July 14 Nos. 5 and 6 injected with saline suspension of testis.
† On Sept. 13 No. 5 injected with saline suspension of testis. Nos. 3 and 6 injected with saline suspension of brain.

dates refer to the first of five 24-hour periods in each case. The mean of the daily creatinine output in mg. is given for each series of 24-hour periods. Rabbits Nos. 2, 3 and 5 were allowed to run free for about 4 months when they were returned to the metabolism cages.

Discussion.

The post-castration rise in urinary creatinine commencing 3 to 4 months after removal of the testes confirms the results reported previously. Examination of the data shows further that injection of saline suspensions of rabbit's or bull's testis led after a slight rise to a fall in creatinine to the pre-castration level. On the other hand, injections of brain suspension had no such effect on either the normal or castrated animal. With rabbits' testes the effect persisted for two days, after which the creatinine rose to the castration level. With bull's testis the fall occurred on the second day after injection after which there was again a rapid increase.

Injection of testicular extracts also caused a decrease to the normal pre-castration level. In rabbit No. 4 the final injection on July 28th led to a creatinine output for that day which was below normal. The decrease persisted throughout the injection periods, rising again when injections ceased. Injections of brain extract or of olive oil had no effect. Rabbit No. 3, a normal animal, was injected on July 28th with testicular extract instead of with olive oil and this led to an immediate drop in creatinine excretion.

The decrease in the high creatinine of castration caused by injections of testicular suspensions or extracts and the subsequent post-injection rise are effects which are in complete agreement with those obtained with grafting of testes and subsequent removal of the graft.

The results as a whole thus afford strong evidence in favour of the existence of an endocrine relationship between the testes and creatinine metabolism. This relationship may be a direct one or it may be an indirect one through some other organ of internal secretion. The fact that the castration effect only appears after a latent period of several months suggests that it is the result of a slow, gradual change in another endocrine organ. This hypothesis is supported by the results of recent work on the relation between the gonads and the pituitary and the pituitary and creatinine metabolism. Martins and Rocha [1931] review the evidence in regard to the regulation of pituitary activity by the testes and report their results on parabiotic experiments in rats. They come to the conclusion that as a result of castration a pituitary hypertrophy, associated with the presence of "castration cells" in the anterior lobe, occurs and the organism becomes flooded with anterior pituitary hormones. The gonads normally exert an inhibitory effect on the pituitary gland.

Braier [1931] has shown that hypophysectomised dogs on a complete diet eliminate 24 % less creatinine than the controls. This difference is accentuated

in fasting animals, removal of the pituitary leading to a 35–40 % decrease in creatinine excretion. This effect was obtained as early as 30 days after operation. Evidence in regard to the effect of injections of pituitary extracts on urinary creatinine is lacking but it is reasonable to assume on the basis of these results that increased activity of the pituitary gland associated with hypertrophy of the anterior lobe occurring as the result of castration would lead to an increased elimination of creatinine.

On the assumption that castration causes marked hypertrophy of the anterior lobe of the pituitary in male rabbits the castration effect on creatinine excretion is explicable as being due to the unantagonised hyperactivity of the anterior lobe.

The continuous stimulating effect of the pituitary is rapidly and effectively antagonised by injection of saline suspensions of testes or testicular extracts and more slowly but as effectively by grafting testes into castrated animals. In both cases the creatinine excretion drops to normal. The effect is a transient one with injection but persists as a result of grafting. Removal of the graft almost immediately brings into operation the effect of pituitary hyperactivity and the creatinine rises to the typical castration level; the same effect is seen during the post-injection periods.

SUMMARY.

1. Injection of saline suspensions of testes or of testicular extracts causes the high urinary creatinine of castration to decrease to the normal pre-castration level.

2. It is pointed out that the results reported above together with those in a previous paper afford strong evidence in favour of the existence of an endocrine relationship between the testes and creatinine metabolism.

3. It is suggested that the castration effect is an indirect one through the anterior lobe of the pituitary gland.

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CCXXV. SOME NEW ANTISEPTICS RELATED TO INDAN-1:3-DIONE.

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(*Received September 29th, 1932.*)

Many compounds containing the 1 : 3-diketo-system, or the enolic form of the same, have been found to possess definite forms of physiological activity, and such properties may be particularly evident in molecules in which the 1 : 3-diketo-grouping constitutes part of a ring structure to which one or more alkyl or acyl groups are attached in certain positions. Cases in point are furnished by the hypnotic derivatives of barbituric acid, in which a *gem*-dialkyl group is situated between the two carbonyls; further instances are those of filicic acid and aspidinol, nuclear units of complex and more active substances present among the constituents of *Aspidium filix-mas* and of other ferns [*cf.* Henry, 1923].

The nucleus of filicic acid contains the grouping

$$\overset{1}{\text{CO}}\text{—}\overset{2}{\text{C}} : (\text{CH}_3)_2\text{—}\overset{3}{\text{CO}}\text{—}\overset{4}{\text{C}}\text{=}$$

and possesses a substituent acyl group attached to carbon atom number 4. Recognition of the presence of such a system in the naturally occurring anthelmintics has led to the examination of the properties of synthetic acyl derivatives of resorcinol and of phloroglucinol [Karrer and Rosenfeld, 1921] and these have been found to possess different degrees of anthelmintic activity.

Alkyl derivatives of resorcinol and of phloroglucinol, particularly a hexyl derivative of the former [Johnson and Lane, 1921; Leonard, 1924; Dohme, Cox and Miller, 1926; Klarmann, 1926; Klarmann and Figdor, 1926] possess marked antiseptic potency and it is noteworthy that lupulon, a highly antiseptic phloroglucinol derivative occurring in the soft resin of hops [Walker, 1924; 1925; Wöllmer, 1925; Wieland, Marz and Hoek, 1925; Wieland and Marz, 1926; Pyman, 1927], bears a very close structural relationship to filicic acid, an additional indication that antiseptic activity of a certain kind and anthelmintic activity are properties connected with the particular type of molecular architecture possessed by these substances.

Consideration of these and of similar relationships led one of us to make further investigation of the extent to which the 1 : 3-diketo-system, in conjunction with other groups, can condition or enhance physiological activity,

particularly antiseptic potency, and recently it was demonstrated [Black, Shaw and Walker, 1931; Walker *et al.*, 1931] that a number of derivatives of indan-1 : 3-dione, produced by attachment of the malonyl or mono-*n*-alkylmalonyl group to the nuclei of phenols, exert considerable bacteriostatic effects upon certain micro-organisms. In three cases it was shown that, starting from monoethylmalonyl chloride, the coupling of an homologous series of mono-*n*-alkylmalonyl chlorides with a given phenol resulted in the production of an homologous series of 2-*n*-alkylindan-1 : 3-diones of progressively increasing antiseptic power; and in the series derived from *p*-cresol such increase in power attained its limit with the compound containing the *n*-heptyl radical.

In view of this behaviour and of the fact that similar progressive increase of antiseptic potency has been noted in the homologous series of *n*-alkylresorcinols, where it culminates in the *n*-hexyl compound [Dohme, Cox and Miller, 1926; Klarmann, 1926], it appeared probable that condensations of *n*-amyl- and *n*-hexylmalonyl chlorides with suitable *n*-amyl, *n*-hexyl and *n*-heptyl derivatives of phenol would give rise to indan-1 : 3-diones of considerably greater antiseptic potency than those hitherto prepared.

Accordingly, a series of *p*-*n*-alkylphenols was synthesised and coupled with various *n*-alkylmalonyl chlorides, and investigation of the properties of the products has fully justified expectations as to their degrees of antiseptic activity. It was also intended originally to apply this reaction for the production of indan-1 : 3-diones to *C*-alkyl derivatives of *o*-, *m*-, and *p*-cresol but, whilst experiments with 3-*n*-amyl-*p*-cresol and indan-1 : 3-diones derived from it were in progress, Coulthard, Marshall and Pyman [1930] published an account of the syntheses and germicidal activities of several homologous series of phenols, including *C*-alkyl derivatives of *o*-, *m*- and *p*-cresol, and the data given in their paper have rendered unnecessary further work on the lines we had in view.

The constitutions of the new indan-1 : 3-diones and the numbers assigned to them for reference purposes are shown in Tables II and III, compounds I to XIV, inclusive, possessing structure (A), and compounds XV to XVIII, inclusive, possessing structure (B). Compound (I) was prepared during the course of a previous investigation [Walker *et al.*, 1931] and is included here in order to complete series I to VII.

The new substances exercise selective antiseptic action, for whilst they inhibit, even in very low concentrations, the proliferation of many Gram-positive bacilli and cocci, they exert little or no effect on the reproduction of certain Gram-negative organisms, relatively large doses of the substances failing to restrain the development of *Bact. coli commune*, *Bact. pyocyaneum* and *Bact. prodigiosum*, in broth or other media.

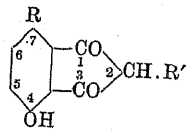
Table I permits comparison of the bacteriostatic powers of the members of the series of *p*-*n*-alkylphenols and of that of 3-*n*-amyl-*p*-cresol, whilst comparisons of the powers of the new indan-1 : 3-diones prepared from these substances may be made from the data in Tables II and III. The inhibiting

Table I.

Substance	Bacteriostatic concentration expressed as 1 g. of substance in x cc. of medium	Equimolecular phenol coefficient of bacteriostatic power	Equimolecular phenol coefficient of bactericidal power (calculated from phenol coefficients obtained by use of Rideal-Walker method)
Phenol	1 in 660	—	—
p-Cresol	1 in 1,580	2·7	2·87
p-Ethylphenol	1 in 1,660	3·2	9·7
p-*n*-Propylphenol	1 in 8,300	16·2	28·9
p-*n*-Butylphenol	1 in 25,000	59·8	111·7
p-*n*-Amylphenol	1 in 76,900	189	181·4
p-*n*-Hexylphenol	1 in 125,000	355	170·4
p-*n*-Octylphenol	1 in 550,000	1827	—
3-*n*-Amyl-*p*-cresol	1 in 71,400	205	473

[The concentrations of *p*-*n*-amylphenol and *p*-*n*-hexylphenol necessary to suppress the proliferation of *Bact. coli commune* in broth (p_H 6·1) for 48 hours at 37° were found to be 1 in 8300 and 1 in 12,500, respectively, whilst under the same conditions and in the same space of time either compound at a concentration of 1 in 5000 was found to kill this organism. A concentration of 1 in 100,000 of *p*-*n*-octylphenol killed *Bacterium C* in 48 hours but *Bact. coli commune* was not killed in 48 hours by this compound at a concentration of 1 in 25,000 (saturated solution).]

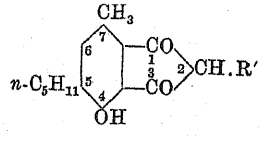
Table II.



(A)

		Bacteriostatic concentration, expressed as 1 g. of substance in x cc. of medium	Equimolecular phenol coefficient of bacteriostatic power
(I)	R=CH_3	1 in 45,000 { 1 in 9,080 at p_H 6·7 ; 1 in 3,630 at p_H 7·7 }	170
(II)	R=C_2H_5	1 in 125,000	490
(III)	R=n-C_3H_7	1 in 222,000	920
(IV)	R=n-C_4H_9	1 in 555,000	2,430
(V)	R=n-C_5H_{11}	1 in 1,250,000	5,740
(VI)	R=n-C_6H_{13}	1 in 3,333,000	16,060
(VII)	R=n-C_8H_{17}	1 in 5,000,000	26,330
(VIII)	R=n-C_4H_9	1 in 50,000 { 1 in 20,000 at p_H 6·7 ; 1 in 6,660 at p_H 7·7 }	196
(IX)	R=n-C_6H_{13}	1 in 500,000	2,187
(X)	R=n-C_5H_{11}	1 in 3,333,000	16,060
(XI)	R=n-C_6H_{13}	1 in 5,000,000 { 1 in 2,500,000 at p_H 6·7 ; 1 in 500,000 at p_H 7·7 }	25,212
(XII)	R=C_2H_5	1 in 250,000 { 1 in 125,000 at p_H 6·7 ; 1 in 62,500 at p_H 7·7 }	1,093
(XIII)	R=n-C_5H_{11}	1 in 1,000,000	5,044
(XIV)	R=n-C_6H_{13}	1 in 5,000,000 { 1 in 1,250,000 at p_H 6·7 ; 1 in 833,300 at p_H 7·7 }	26,330

Table III.



(B)

		Bacteriostatic concentration, expressed as 1 g. of substance in x cc. of medium	Equimolecular phenol coefficient of bacteriostatic power
(XV)	R'=n-C_3H_7	1 in 833,300	3,830
(XVI)	R'=n-C_4H_9	1 in 1,666,600	8,030
(XVII)	R'=n-C_5H_{11}	1 in 3,333,000	16,800
(XVIII)	R'=n-C_6H_{13}	1 in 250,000	1,330

concentrations were determined by the turbidity method in lemco-peptone broth (p_H 6·1, unless otherwise stated), the organism employed being *Bacterium C* (National Collection of Type Cultures, Lister Institute). Incubation in the presence of different doses of each antiseptic was allowed to proceed for 48 hours at 37° and the results of all the tests were confirmed by triplicate determinations. *Bacterium C* was selected originally for the purpose after preliminary trials had shown that its behaviour towards this class of antiseptics is representative of that of a number of Gram-positive bacilli and cocci; moreover *Bacterium C* develops rapidly, it may be cultured over considerable periods of time without change of properties, and, in other ways, it is very suitable for use in determinations of bacteriostatic power.

In Tables I, II and III, the third column in each case shows the bacteriostatic powers of the several compounds compared with that of phenol and expressed on an equimolecular basis[1], *i.e.* equimolecular phenol coefficient of bacteriostatic power = (phenol coefficient of bacteriostatic power × molecular weight of substance) ÷ molecular weight of phenol. The figures in the fourth column of Table I have been calculated from phenol coefficients given by Coulthard, Marshall and Pyman [1930], and they are shown here for purposes of comparison. It is of interest to note in the case of the *p-n*-alkylphenols that while both the bacteriostatic activity towards *Bacterium C* and the bactericidal activity towards *Bact. typhosum* increase with the number of carbon atoms in the side-chain, the bactericidal activity attains its maximum in the *p-n*-amyl derivative whereas the bacteriostatic activity continues to increase along the series beyond the *p-n*-amyl derivative and may not have attained its maximum in the *p-n*-octyl derivative, the highest member examined. The *p-n*-heptyl derivative of phenol was not prepared since oenanthic acid was not available at the time this work was undertaken.

Reference has already been made to the fact that in the homologous series of 4-hydroxy-7-methyl-2-*n*-alkylindan-1 : 3-diones antiseptic activity was found to increase progressively until it attained a maximum intensity in the 2-*n*-heptyl derivative. Similar behaviour is to be noted in two homologous series of this type shown in Tables II and III, respectively. In Table II, compounds IX, VI, XI and XIV are members of such an homologous series and it would appear that the antiseptic activity has culminated in XIV, where R' = *n*-hexyl, since the equimolecular phenol coefficient of this compound is only very slightly greater than that of XI. The four compounds in Table III show very definitely the same kind of behaviour; here the 2-*n*-amyl derivative possesses the greatest antiseptic power, the 2-*n*-hexyl compound proving to be relatively weak.

Scrutiny of Table II also shows that progressive increase of antiseptic potency occurs in the series of compounds prepared by coupling a given alkylmalonyl chloride with an homologous series of *p-n*-alkylphenols, and if

[1] Morgan, Cooper and Burtt [1923] have pointed out that it is preferable to compare phenol coefficients on an equimolecular basis.

we consider the series I to VII, inclusive, it is seen that bacteriostatic potency is very considerably higher in the case of the 7-*n*-octyl derivative VII than in that of the 7-*n*-hexyl derivative VI. Since the 7-*n*-heptyl and the 7-*n*-nonyl derivatives were not prepared, it is not possible to decide from the present data whether the 7-*n*-octyl derivative is the most potent compound of this type which could be synthesised. Further synthetical work in this series was not practicable, however, for great difficulty was encountered in separating pure substances from the highly resinous reaction products which were obtained when components containing these larger alkyl groups were employed in the condensation.

Bacteriostatic and bactericidal action of indan-1:3-diones on a variety of micro-organisms.

In order to obtain information on broader lines concerning the inhibitory and lethal effects of the new indan-1 : 3-diones, three of these compounds (*viz.* X, XIV and XVII) which, in tests against *Bacterium C*, had exhibited considerable bacteriostatic powers, were employed in experiments with the following organisms: *Bact. pyocyaneum* Flügge (Fildes III), *Bact. prodigiosum* Cohn, *Bact. coli* Escherich, *Staphylococcus albus* Rosenbach, *Bac. megatherium* de Bary, *Bac. subtilis* (Ehrenberg) Cohn, *Bac. mycoides* Flügge and two strains of *Mycobact. phlei* (Möller) Lehmann and Neumann. These organisms, with the exception of the specimen of *S. albus*, were obtained from the National Collection of Type Cultures, Lister Institute.

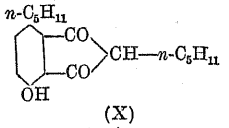
The determinations of bacteriostatic and bactericidal activity were made by the turbidity method previously employed in the case of *Bacterium C*, living organisms being sought for in subcultures when visual inspection of the tubes failed to detect growth. The results are shown in Table IV where, for purposes of comparison, the effects of these substances on *Bacterium C* are also reproduced.

These determinations have served to emphasise the highly selective action of the substances, since *Bact. pyocyaneum*, *Bact. prodigiosum* and *Bact. coli* were not prevented from multiplying by concentrations many times greater than those found necessary to kill the organisms cited in Table IV. Marked selectivity is also seen in the cases of *S. albus* and *M. phlei*, compound X exerting intense bacteriostatic effects upon the former organism, while the latter organism appears to be particularly susceptible to the influence of compound XIV.

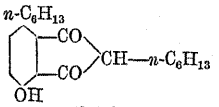
It was not considered necessary to determine the exact bactericidal concentration in more than half of the cases studied; in other instances it was ascertained whether the lethal dose lay above or below a given figure. The data shown in Table IV are adequate, however, to indicate that in some cases (*e.g.* that of *M. phlei*) the dose of antiseptic which is just sufficient to inhibit growth over a period of 48 hours is also lethal in that time, that is to say, in such cases the germicidal concentration is only slightly higher than the highest

Table IV. *Results illustrating the bacteriostatic and bactericidal activities of indan-1:3-dione derivatives towards various micro-organisms.*

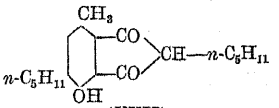
(*a*) = bacteriostatic concentration in broth (p_H 6·1) at 37° for 48 hours; (*b*) = bactericidal concentration in broth (p_H 6·1) at 37° for 24 hours.



(X)



(XIV)



(XVII)

Organism	(X) (*a*)	(X) (*b*)	(XIV) (*a*)	(XIV) (*b*)	(XVII) (*a*)	(XVII) (*b*)
**Bacterium C*	1 : 3,333,000	>1 : 166,000	1 : 5,000,000	<1 : 166,000	1 : 3,333,000	<1 : 166,000
Bac. megatherium	1 : 2,500,000	>1 : 500,000	—	—	1 : 2,500,000	>1 : 1,666,000
Bac. subtilis	1 : 2,500,000	<1 : 500,000	1 : 5,000,000 (1 : 1,250,000 at p_H 7·7)	1 : 1,430,000 (1 : 833,000 at p_H 7·7)	1 : 2,500,000	>1 : 1,000,000
Bac. mycoides	1 : 2,500,000	<1 : 500,000	1 : 5,000,000 (1 : 1,250,000 at p_H 7·7)	1 : 2,000,000 (1 : 1,250,000 at p_H 7·7)	1 : 3,333,000	1 : 1,666,000
S. albus	<1 : 10,000,000	<1 : 500,000	1 : 3,333,000 (1 : 1,000,000 at p_H 7·7)	1 : 1,666,000 (1 : 833,000 at p_H 7·7)	1 : 2,500,000	1 : 1,666,000
M. phlei	(i)	(i)	(i)	(i)	(i)	(i)
(i) strain 525	1 : 5,000,000	<1 : 500,000	1 : 11,110,000	1 : 11,110,000	1 : 3,333,000	1 : 3,333,000
(ii) strain 54			(ii) 1 : 20,000,000 (ii) (1 : 1,428,000 at p_H 7·7)	(ii) 1 : 20,000,000 (ii) (1 : 1,428,000 at p_H > 7·7)	(ii) 1 : 3,333,000	(ii) 1 : 2,000,000

* This unclassified organism was designated provisionally as *Bacterium C* by Chapman but, since it gives rise to spores it could be designated, according to the convention of Lehmann and Neumann, as *Bacillus C.*

concentration of antiseptic in the presence of which growth can occur with comparative freedom. In other cases, according to the particular organism, the concentration of antiseptic necessary to produce germicidal effects within 24 hours may be as much as 20 times higher than that which is sufficient to inhibit growth during the same period (for example, *Bacterium C* and compound X).

In view of these results it was thought of interest to obtain further information as to the intensity of the bactericidal activity of the substances towards Gram-positive organisms and, accordingly, the action of compound XI on *Bac. megatherium*, *Bac. subtilis*, *Bac. mycoides* and *S. albus* was studied under the conditions of the Rideal-Walker method. The substance (0·1 g.) was first dissolved in 100 cc. of 0·02 *N* Na_2CO_3 and, by dilution of aliquot portions of this stock solution, with sterile water, the concentrations required were obtained (Table V).

The bactericidal action of these indan-1 : 3-diones is seen, therefore, to be rapid at higher concentrations, and still reasonably rapid even at low concentrations, and, generally, in their antiseptic effects the substances are strikingly similar to the triphenylmethane dyes of the type of crystal violet which, as Churchman [1926] has shown, are much more powerfully bacteriostatic towards Gram-positive organisms such as staphylococcus, diphtheroids and

Table V.

Organism	Time, in minutes, necessary for completion of the lethal action of compound XI at concentrations of 1 : 10,000	1 : 100,000	1 : 1,000,000
Bac. megatherium	<10	—	<30
Bac. subtilis	<10	<30	—
Bac. mycoides	<10	<60	—
S. albus	<10	—	<60

Bact. coli was killed in 30 minutes but not in 15 minutes by compound XI at a concentration of 1 : 1000.

aerobic spore-forming bacilli than towards Gram-negative organisms like *Bact. coli*. With the indan-1 : 3-diones, however, this selectivity of action is decidedly more pronounced than with the triphenylmethane dyes, and this fact would appear to indicate that the former compounds could be utilised very successfully in the separation of coliform organisms from certain mixed cultures.

The effect of hydrogen ion concentration on the antiseptic activity of indan-1:3-diones.

Tables II and IV contain sufficient data concerning the effects of hydrogen ion concentration to indicate clearly that the antiseptic activities of the substances examined are greatly diminished by adjustment of the medium from the acid range of the scale towards the neutral point, and that this effect becomes still more marked when the medium is rendered faintly alkaline. It is also evident that no general statement can be made regarding the relative effects likely to be produced by alterations in the reaction of the medium, since two compounds which exercise approximately equal inhibitory actions upon a given organism at one particular hydrogen ion concentration, may manifest widely divergent activities towards the same organism at other hydrogen ion concentrations.

The effect of serum on the antiseptic activity of indan-1:3-diones.

It was considered desirable to ascertain the effect of serum upon the bactericidal activities of indan-1 : 3-dione derivatives, and Prof. C. H. Browning very kindly undertook to examine one of the new compounds from this point of view. The substance chosen was 4-*hydroxy*-7-n-*hexyl*-2-n-*amyl indan*-1 : 3-*dione* (compound XI) which, as previous tests had shown, is one of the most potent of the new compounds. He reported as follows:

"0·05 g. was dissolved in 5 cc. alcohol and 5 cc. water added; the precipitate which formed was dissolved by the addition of a drop of Na_2CO_3 solution. This 0·5 % stock solution was stable and from it further dilutions in water were made. A volume not exceeding 0·1 cc. of various dilutions was then added to a series of tubes each containing 1 cc. 0·7 % peptone water medium or ox-serum (previously heated at 56°), so as to yield a wide range of concentrations of the antiseptic. In peptone water precipitation occurred

with 1 : 20,000 of the substance, but 1 : 2000 in serum gave no precipitate. The tubes were then inoculated with 0·1 cc. of a 1 : 1000 dilution in saline of a 24 hours' fluid culture of *S. aureus* or *Bact. coli*. The results were read after 48 hours' incubation at 37°, both naked-eye evidence of growth and the presence of living organisms, as tested by subculture, being determined.

The *Staphylococcus* in peptone water was sterilised by 1 : 400,000, but in serum 1 : 2000 failed to sterilise. In the case of *Bact. coli*, 1 : 2000 failed to sterilise in peptone water and in serum."

Syntheses of acylphenols and reduction of these to alkylphenols.

The *p*-*n*-alkyl derivatives of phenol examined by Coulthard, Marshall and Pyman [1930] were obtained by reduction of the corresponding *p*-acylphenols by Clemmensen's method. These authors prepared *p*-propionyl, *p*-*n*-valeryl and *p*-*n*-hexoylphenols by heating phenol with the appropriate fatty acid and anhydrous zinc chloride, a process due originally to Nencki. We found that much better yields of these three ketones were obtained by employing the Fries isomerisation, a method used by Coulthard, Marshall and Pyman [1930] to obtain the corresponding derivatives of the three cresols. In our experiments the acid chloride (1 mol.) was added to phenol (1 mol.) and 30 minutes later the mixture was warmed on a water-bath for 60 minutes. The resulting ester of the phenol was then diluted with three times its volume of freshly distilled nitrobenzene, and aluminium chloride (1·1 mols.) in a finely powdered condition was added in small quantities at a time. The reaction was completed by heating on a water-bath for 60 minutes. The material was then submitted to steam-distillation in order to remove nitrobenzene and to separate the volatile *o*-hydroxyarylketone from the non-volatile *p*-isomeride. The latter was isolated by extraction with ether and distillation under low pressure. The yields were: *p*-propionylphenol, 25 %; *p*-*n*-butyrylphenol, 18 % (Coulthard, Marshall and Pyman obtained 19 % in absence of nitrobenzene); *p*-*n*-valerylphenol, 37 %; *p*-*n*-hexoylphenol, 58 %.

The preparation of 3-*n*-valeryl-*p*-cresol (65 % yield) was carried out under the conditions used by Coulthard, Marshall and Pyman, *i.e.* the isomerisation was conducted at 160° in absence of nitrobenzene.

p-Acetylphenol was prepared according to a method described by Nencki [1897], which consisted in treating a mixture of phenol and acetyl chloride in carbon disulphide solution with ferric chloride. The yield was 60 %. The reduction of the above-mentioned *p*-hydroxyarylketones to the corresponding alkylphenols by Clemmensen's method was effected in every case with a yield of 80 % or over.

p-*n*-Octylphenol has been described recently by Sandulesco and Girard [1930]. We prepared it as follows. *n*-Caprylic acid (73 g.) on treatment with thionyl chloride (88 g.) gave 57 g. (70 % yield) of the acid chloride, which boiled at 101–102°/18 mm. This was added to freshly distilled phenol (33 g.) and, after the resulting reaction had moderated, the mixture was warmed for

60 minutes on a steam-bath and subsequently diluted with 200 cc. of freshly distilled nitrobenzene. Finely powdered anhydrous aluminium chloride (52 g.) was now added in small portions, and the reaction was completed by heating for an hour at 75°. The reaction product was poured on ice, treated with hydrochloric acid and submitted to steam-distillation. The non-volatile organic residue was taken up in ether and was subsequently fractionated under reduced pressure. Some material which distilled up to 180° at 13 mm. probably contained a little *o-n*-caprylylphenol; the *p-n*-caprylylphenol subsequently passed over at 215 to 220°/15 mm. and solidified on standing. This crude fraction (30 g., *i.e.* 40 % yield) was not further purified but was reduced by heating with 100 g. of amalgamated zinc-wool and 600 cc. of dilute hydrochloric acid (1 vol. concentrated acid : 2 vols. water) on a water-bath at 100° during 16 hours, the acid being added in portions from time to time. (The zinc-wool had been amalgamated by treatment for 5 minutes with a 5 % aqueous solution of mercuric chloride.) The reduction product, isolated by extraction with ether, was distilled under reduced pressure and the fraction boiling at 195–205°/15 mm. (Sandulesco and Girard give 169°/10 mm.) was collected. This crystallised in colourless needles, on standing, and the yield was 55 %. Owing to the fact that the melting-point was low and indefinite (*ca.* 41°) the substance was characterised as the p-*nitrobenzoate*, which was prepared by heating 3 g. of the phenol with 3 g. of *p*-nitrobenzoyl chloride in 20 cc. of pyridine on a water-bath for 2 hours. The reaction mixture was poured into dilute hydrochloric acid and the solid product was separated, dried and crystallised from acetone. It melted at 81·5°. (Found, by micro-method: N, 4·07 %, $C_{21}H_{25}O_4N$ requires N, 3·94 %.)

A communication by Sandulesco and Girard [1930], which was published almost simultaneously with that of Coulthard, Marshall and Pyman, also contains an account of the syntheses of *o*- and *p*-acyl derivatives of phenol and of their subsequent reduction to the corresponding alkyl derivatives, the methods employed being very similar to those used independently by us and by Coulthard, Marshall and Pyman. Sandulesco and Girard state that somewhat higher yields of the *p*-acyl derivatives of phenol are obtained when working in the absence of nitrobenzene. Their yields of *p*-alkyl derivatives of phenol obtained by use of Clemmensen's reduction method are of the same order as those obtained by us.

Syntheses of 4-hydroxyindan-1:3-diones.

A general method, and modifications of the same, for the preparation of substances of this class were described in a former communication [Walker *et al.*, 1931]. The 17 new compounds examined in the course of the present investigation were all prepared by this same general process, namely, condensation of an alkylmalonyl chloride with an alkylphenol in nitrobenzene solution in the presence of anhydrous aluminium chloride. In some cases, however, difficulties were encountered in purifying the product, and this was

the case notably in those instances where R and R' were alkyl groups containing more than four carbon atoms. Increase in length of the alkyl chain in the malonyl chloride component was accompanied by an increasing tendency to resin formation, particularly if the reaction in nitrobenzene solution was conducted above 80° and, on the other hand, the employment of a temperature lower than 70° resulted in a low yield since a smaller percentage of the components interacted. In this latter event the product was contaminated with relatively large amounts of alkylmalonic acid and unchanged alkylphenol, and the latter, owing to low volatility, could not be removed in steam. In such cases the following procedure was adopted for the isolation and purification of the indan-1 : 3-dione. The nitrobenzene solution of the reaction products was poured on to ice and treated with sufficient hydrochloric acid to take the aluminium into solution. The nitrobenzene was removed in steam and the cooled residue was extracted thoroughly with ether. The ethereal extract was then washed many times with water to remove all traces of alkylmalonic acid, after which it was extracted very thoroughly with successive portions of a large bulk of a 7·5 % aqueous solution of sodium carbonate, followed by an extraction with cold water. This removed the indan-1 : 3-dione in the form of its sodium salt, and unchanged alkylphenol was left in the ether. The combined sodium carbonate extracts together with the final aqueous extract were acidified with hydrochloric acid and the free indan-1 : 3-dione was taken up in ether when, after drying and removal of the ether, it solidified and was recrystallised from appropriate solvents.

All the new indan-1 : 3-diones are colourless solids which are practically insoluble in cold water and soluble in aqueous solutions of alkali metal hydroxides or alkali metal carbonates. They are generally soluble in, and crystallise well from, the usual organic solvents, the most suitable of which for purposes of purification were found to be acetone and methyl alcohol in the hot, and an acetone-light petroleum mixture.

The analytical data and melting-points are given in Table VI. The combustions were performed by the micro-method.

Table VI.

Substance (see Tables II and III)		Formula	M.P.	Found % C	Found % H	Calculated % C	Calculated % H
4-Hydroxy-7-ethyl-2-*n*-butylindan-1:3-dione	(II)	$C_{15}H_{18}O_3$	111°	73·0	7·5	73·2	7·3
4-Hydroxy-7-*n*-propyl-2-*n*-butylindan-1:3-dione	(III)	$C_{16}H_{20}O_3$	122–123	74·5	8·1	73·8	7·7
4-Hydroxy-2:7-di-*n*-butylindan-1:3-dione	(IV)	$C_{17}H_{22}O_3$	120	74·9	7·9	74·4	8·0
4-Hydroxy-7-*n*-amyl-2-*n*-butylindan-1:3-dione	(V)	$C_{18}H_{24}O_3$	110	75·2	8·4	75·0	8·3
4-Hydroxy-7-*n*-hexyl-2-*n*-butylindan-1:3-dione	(VI)	$C_{19}H_{26}O_3$	118–119	76·0	8·7	75·5	8·6
4-Hydroxy-7-*n*-octyl-2-*n*-butylindan-1:3-dione	(VII)	$C_{21}H_{30}O_3$	105	76·4	9·35	76·4	9·1
4-Hydroxy-7-*n*-butyl-2-ethylindan-1:3-dione	(VIII)	$C_{15}H_{18}O_3$	129	73·2	7·3	73·2	7·3
4-Hydroxy-7-*n*-hexyl-2-ethylindan-1:3-dione	(IX)	$C_{17}H_{22}O_3$	120	74·1	8·4	74·4	8·0
4-Hydroxy-2:7-di-*n*-amylindan-1:3-dione	(X)	$C_{19}H_{26}O_3$	114–115	75·2	9·0	75·5	8·6
4-Hydroxy-7-*n*-hexyl-2-*n*-amylindan-1:3-dione	(XI)	$C_{20}H_{28}O_3$	100	75·8	8·9	75·9	8·8
4-Hydroxy-7-ethyl-2-*n*-hexylindan-1:3-dione	(XII)	$C_{17}H_{22}O_3$	110	74·6	7·9	74·4	8·0
4-Hydroxy-7-*n*-amyl-2-*n*-hexylindan-1:3-dione	(XIII)	$C_{20}H_{28}O_3$	98–99	75·5	9·0	75·9	8·8
4-Hydroxy-2:7-di-*n*-hexylindan-1:3-dione	(XIV)	$C_{21}H_{30}O_3$	95–96	76·0	9·3	76·4	9·1
4-Hydroxy-5-*n*-amyl-7-methyl-2-*n*-propylindan-1:3-dione	(XV)	$C_{18}H_{24}O_3$	149	75·5	8·0	75·0	8·3
4-Hydroxy-5-*n*-amyl-7-methyl-2-*n*-butylindan-1:3-dione	(XVI)	$C_{19}H_{26}O_3$	119–120	76·1	8·9	75·5	8·6
4-Hydroxy-7-methyl-2:5-di-*n*-amylindan-1:3-dione	(XVII)	$C_{20}H_{28}O_3$	90–92	76·1	8·6	75·9	8·8
4-Hydroxy-5-*n*-amyl-7-methyl-2-*n*-hexylindan-1:3-dione	(XVIII)	$C_{21}H_{30}O_3$	143–144	76·3	9·1	76·4	9·1

SUMMARY.

1. Seventeen new alkyl derivatives of 4-hydroxyindan-1 : 3-dione, all of which possess antiseptic power, have been prepared by the condensation of mono-*n*-alkylmalonyl chlorides with *p*-*n*-alkyl derivatives of phenol and with 3-*n*-amyl-*p*-cresol, in the presence of aluminium chloride.

2. Examination of these products shows that when a given mono-*n*-alkylmalonyl chloride is condensed with the lower members of the homologous series of the *p*-*n*-alkyl derivatives of phenol, the antiseptic powers of the members of the resulting homologous series of 4-hydroxyindan-1 : 3-diones increase progressively as the series is ascended. The limits of such increases have not been determined in the present investigation.

3. Two sets of results now obtained confirm a former observation that the condensation of several consecutive lower members of the homologous series of mono-*n*-alkylmalonyl chlorides with an appropriate phenol results in the formation of an homologous series of hydroxyindan-1 : 3-diones, the members of which exhibit antiseptic power which increases progressively to a maximum value as the series is ascended. Such maximum value appears usually to be attained in the 2-*n*-amyl-, 2-*n*-hexyl-, or 2-*n*-heptyl-compound.

4. The antiseptic activity of these 4-hydroxyindan-1 : 3-diones is highly selective in character. In very low concentrations they inhibit the proliferation of Gram-positive bacilli and cocci, as shown by tests against *Bac. megatherium*, *Bac. subtilis*, *Bac. mycoides*, *S. albus* and *S. aureus*. At higher concentrations the substances exercise lethal action on all these organisms. The acid-fast organism *Mycobact. phlei* is particularly sensitive to the presence of certain of them; thus one strain, in broth (p_H 6·1), is killed within 24 hours at 37° when 4-hydroxy-2 : 7-di-*n*-hexylindan-1 : 3-dione is present at a concentration of 1 : 20,000,000. Another strain of the same organism is killed under the same conditions when the same compound is present at a concentration of 1 : 11,110,000.

On the other hand these compounds have little or no adverse action on the proliferation, in broth, of the Gram-negative organisms *Bact. coli*, *Bact. pyocyaneum* and *Bact. prodigiosum*.

5. The antiseptic activities of the new substances are depressed considerably by culture media which are slightly alkaline in reaction, and are suppressed entirely by ox-serum.

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CCXXVI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXV. 3:5-DIHYDROXYPHTHALIC ACID, A NEW PRODUCT OF THE METABOLISM OF GLUCOSE BY *PENICILLIUM BREVI-COMPACTUM* AND RELATED SPECIES.

BY ALBERT EDWARD OXFORD AND HAROLD RAISTRICK.

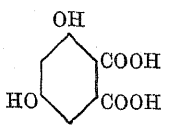
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IN Part XXIV of this series [Clutterbuck, Oxford, Raistrick and Smith, 1932] it was shown that out of 15 species in the *P. brevi-compactum* series submitted to examination, 14 metabolised glucose into a mixture of phenolic acids from which were isolated four new substances with the empirical formulae $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$ respectively. The last-named was detected with certainty in 10 cases only, but since the yield of this substance actually isolated was never greater than 0·03 % of the sugar metabolised, being always much smaller than that of any of the other metabolic products, and since the method for its isolation and separation from tarry and other impurities inevitably led to considerable losses, it appears quite likely that it was in fact produced in all 14 instances. This paper deals with the constitution of this compound $C_8H_6O_6$, which, remarkably enough, is isomeric with puberulic acid [Part XXIII, Birkinshaw and Raistrick, 1932], a metabolic product of *P. puberulum* Bainier and *P. aurantio-virens* Biourge, the constitution of which is not yet completely elucidated, although, in view of what follows later it is of interest that these authors consider that it is probably not a dihydroxybenzenedicarboxylic acid (Part XXIII, 1932, pp. 447–9).

It was recorded in Part XXIV (p. 1455) that the new compound $C_8H_6O_6$ loses nearly 1 molecule of water (together with some CO_2) at its first M.P. (188–190°), titrates sharply as a dibasic acid, and gives a stable deep red coloration with ferric chloride and a magenta coloration with bleaching powder solution. It has since been found that complete methylation with diazomethane yields a crystalline tetramethyl derivative which is insoluble in cold alkali and gives no coloration with ferric chloride. These facts not only make it probable that $C_8H_6O_6$ is a dihydroxybenzenedicarboxylic acid, but also point clearly to one particular acid in that series, since the only dihydroxy-

benzenedicarboxylic acid which would behave as a true phthalic acid on heating and also exhibit the colour reactions of β-resorcylic acid is the unknown *3:5-dihydroxyphthalic acid* (I). The dimethyl and diethyl ethers of this acid



(I)

and their anhydrides are, however, known [Fritsch, 1897], having been synthesised by a series of reactions leaving no doubt as to their constitution. Asahina and Asano [1932] have also synthesised 3:5-dimethoxyphthalic anhydride by the same method, for comparison and identification with an oxidation product of dimethylolivetonic acid, the latter being derived, by methylation and opening of a lactone ring, from olivetonide, one of the products of the fission (by dehydration) of the lichen acid olivetoric acid extracted from the lichen *Alectoria divergens*.

The dimethyl ether of our mould metabolic product was readily obtained by hydrolysis of the tetramethyl derivative by hot alkali, and was found to crystallise from water with $1H_2O$ (lost below 100°) and to lose water of constitution at 157–158°, to yield the corresponding anhydride, M.P. 147–149°. All these facts are in complete agreement with those recorded by Fritsch [1897] for 3:5-dimethoxyphthalic acid (M.P. 158°) and anhydride (M.P. 147°). The fully ethylated derivative was an oil which lost only one ethyl group when hydrolysed by hot dilute alkali, but the resulting monoethyl ester of the diethyl ether lost the elements of ethyl alcohol at about 180° to yield an anhydride, M.P. 133–134°, a value quite close to 130°, the recorded M.P. of 3:5-diethoxyphthalic anhydride. The identity of our product $C_8H_6O_6$ with 3:5-dihydroxyphthalic acid was finally placed beyond doubt by direct comparison of the anhydride of its dimethyl ether with an authentic specimen of synthetic 3:5-dimethoxyphthalic anhydride very kindly supplied by Prof. Asahina, when a mixed melting-point showed no depression. It is of some interest that this is the first instance in which a constitutional relationship has been definitely established between a mould metabolic product and a group of lichen acids, the structure proposed by Asahina and Asano [1932, p. 477] for olivetoric acid involving the union by loss of H_2O of two fragments, each of which is a 2:4-dihydroxybenzoic acid with a long side chain in the 6-position, and theoretically convertible into 3:5-dihydroxyphthalic acid by oxidation of that side chain. Orsellinic acid, the fundamental unit in the structure of many lichen acids, *e.g.* lecanoric acid, evernic acid, gyrophoric acid, has a similar structure, the side chain in the 6-position being a methyl group. This constitutional relationship is important since it supports the view that it is the fungal part of the fungus-alga symbionts constituting the lichen which is responsible for the formation of the lichen acids.

Experimental.

Properties of the new metabolic product $C_8H_6O_6$ (3:5-dihydroxyphthalic acid).

The general properties of this compound are given in Part XXIV [Clutterbuck, Oxford, Raistrick and Smith, 1932, p. 1455] but are briefly as follows: M.P. 188–190° (efferv.) remelting at 206–210°; crystallises from ethyl acetate-light petroleum in well-formed hexagonal tablets with slow, and in flint-shaped leaflets with rapid, crystallisation. It is readily soluble in cold water and in most organic solvents except light petroleum and benzene. With aqueous or alcoholic ferric chloride it gives a stable deep red colour with perhaps a trace of purple. It gives no precipitate with bromine water. With bleaching powder solution and a dilute solution of the acid in water, a transient magenta colour is obtained. No precipitate is obtained with 2:4-dinitrophenylhydrazine in 2*N* HCl.

Dimethyl ester of the dimethyl ether (dimethyl 3:5-dimethoxyphthalate). 0·4492 g. of the pure substance, which had been recrystallised several times from ethyl acetate-light petroleum, was suspended in dry ether and treated with a large excess of diazomethane prepared from nitrosomethylurethane (4·2 cc.). A vigorous effervescence took place and the solution was allowed to stand for several hours. A few drops were then tested for free phenols by dilution with alcohol and addition of a few drops of dilute ferric chloride solution, when a moderately intense but transient purple coloration was obtained. A further quantity of ethereal diazomethane was therefore added (from 5 cc. nitrosomethylurethane) and the reaction mixture allowed to stand for 2 hours, the ferric chloride test being then negative. The ether was evaporated off and the colourless, crystalline residue (0·6145 g.) was recrystallised from aqueous alcohcl, giving colourless, hair-fine needles, M.P. 92–94°, having a very pronounced ethereal odour.

Micro-analysis (Schoeller): loss at 80° in high vacuum (probably due to slow sublimation) 7·54, 5·87 %. Found on material dried at 80° in high vacuum: C, 56·65, 56·76 %; H, 5·64, 5·71 %; OCH_3, 47·18, 48·08 %; mol. wt. 250, 268. $C_8H_2O_2(OCH_3)_4$ requires C, 56·67 %; H, 5·55 %; OCH_3, 48·83 %; mol. wt. 254.

Dimethyl ether (3:5-dimethoxyphthalic acid). The crude tetramethyl derivative (0·43 g.) was hydrolysed by suspending it in a mixture of 20·0 cc. *N* NaOH and 20 cc. ethyl alcohol and boiling the whole under reflux for 1 hour. The undissolved part (0·03 g.) was then filtered off, and the filtrate exactly neutralised by *N* H_2SO_4 (16·6 cc.), indicating that 3·4 cc. of *N* NaOH had been used up (theoretical, 3·15 cc.). More sulphuric acid was then added and the liquid extracted several times with ether. Evaporation of the dried extract yielded a crystalline deposit which crystallised from boiling water (charcoal) in nearly colourless pointed prisms (0·15 g.), M.P. 157–158° (efferv.), with slight softening previously at about 100°. The melt was taken to 170° and maintained at this temperature till effervescence ceased. It reset immediately on removal from the bath and then re-melted at 143–148°.

Micro-analysis (Schoeller): On hydrated material; C, 49·44, 49·21 %; H, 5·17, 4·98 %; loss at 80° *in vacuo*, 7·45 %. $C_8H_4O_4(OCH_3)_2$, H_2O requires C, 49·16 %; H, 4·96 %; H_2O 7·38 %. On material dried at 80° *in vacuo*: C, 52·44, 52·22 %; H, 4·81, 4·61 %; OCH_3, 27·41, 28·24 %. $C_8H_4O_4(OCH_3)_2$ requires C, 53·08 %; H, 4·45 %; OCH_3, 27·41 %.

0·1474 g. of the hydrated material required 12·17 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 121, the theoretical for $C_8H_4O_4(OCH_3)_2$, H_2O titrating as a dibasic acid, being 122. This ether gives no coloration with ferric chloride either in aqueous or alcoholic solution. It is moderately soluble in cold water and is so readily soluble in cold alcohol that purification by crystallisation from aqueous alcohol, as recommended by Fritsch [1897] leads to considerable loss of material.

Anhydride of the dimethyl ether (*3:5-dimethoxyphthalic anhydride*). The above ether was heated at 170–180° in an oil-bath until effervescence ceased, and the residue purified by sublimation *in vacuo*. The sublimate (micro-needles) melted at 147–149°, and when mixed with a specimen of authentic 3:5-dimethoxyphthalic anhydride, supplied by Prof. Asahina and resublimed by us (M.P. 146–148°) had M.P. 146–149°. Several determinations of the mixed M.P. were carried out.

Micro-analysis (Schoeller): C, 57·64, 57·70 %; H, 3·79, 3·96 %; OCH_3, 29·84, 29·79 %. $C_8H_2O_3(OCH_3)_2$ requires C, 57·66 %; H, 3·87 %; OCH_3, 29·80 %.

Monoethyl ester of the diethyl ether (*monoethyl 3:5-diethoxyphthalate*). 0·5 g. $C_8H_6O_6$ were ethylated by exactly the same procedure given above for methylation, except that nitrosoethylurethane was used in place of nitrosomethylurethane. The product (1·06 g.) was a yellow syrup not completely soluble in alcohol, the alcohol-insoluble portion, a colourless amorphous solid, M.P. 70–80°, being readily soluble in light petroleum. The analytical figures for this latter product correspond closely with those for $(CH_2)_n$, and the hydrocarbon was doubtless formed by polymerisation and decomposition of diazoethane.

Micro-analysis (Schoeller): C, 84·8 %; H, 14·5 %; $(CH_2)_n$ requires C, 85·7%; H, 14·3 %.

The alcohol-soluble ethylation product was hydrolysed by boiling with *N*/2 aqueous-alcoholic NaOH until nearly all had gone into solution. The mixture was then cooled, filtered, acidified with dilute H_2SO_4 and extracted with ether, the extract being dried over anhydrous Na_2SO_4. Evaporation of the ether yielded a yellow, semi-solid paste, which after two recrystallisations from boiling water yielded colourless, long, silky needles, M.P. 97–99° with no effervescence (according to Fritsch 3:5-diethoxyphthalic acid melts at 182° with effervescence). The substance is soluble in cold sodium bicarbonate solution, and gives no coloration with ferric chloride. The following analytical figures (Schoeller) show conclusively that it is a triethyl derivative of $C_8H_6O_6$. Found: C, 59·57 %; H, 6·50 %; OC_2H_5, 47·06 %; $C_8H_3O_3(OC_2H_5)_3$ requires C, 59·55 %; H, 6·42 %; OC_2H_5, 47·88 %.

Anhydride of the diethyl ether (*3:5-diethoxyphthalic anhydride*). The above monoethyl ester showed no tendency to decompose at 140°, but when heated at 180° vigorous effervescence took place, and the odour of ethyl alcohol was perceptible. Heating was continued for 15 minutes after effervescence had ceased, and the residue was sublimed *in vacuo*. The crystalline sublimate had M.P. 133–134°, was insoluble in cold sodium bicarbonate solution, and not very readily soluble in cold caustic potash solution.

Micro-analysis (Schoeller): C, 60·92 %; H, 5·41 %; OC_2H_5, 39·35 %; $C_8H_2O_3(OC_2H_5)_2$ requires C, 60·99 %; H, 5·13 %; OC_2H_5, 38·16 %.

Attempt to isolate 3:5-dihydroxyphthalic anhydride. Pure $C_8H_6O_6$ (0·4 g.) was heated in a stream of nitrogen at 190–200° until a quiet melt was obtained and no more water or CO_2 was evolved. A trace of material had sublimed and condensed on the cool part of the tube. The solid residue, dark in colour, was readily and completely soluble in saturated $NaHCO_3$ solution. This was immediately shaken with ether but it was found that practically nothing was extractable. The solution was acidified and again extracted with ether, the ether extract yielding a solid product which, after crystallisation from chloroform containing a little ethyl acetate, was obtained in the form of needles melting between 210 and 220° with marked softening previously at 190°, and having an equivalent of about 120. The product gave an intense purple-red colour with ferric chloride and was probably a mixture of the original $C_8H_6O_6$ together with α-resorcylic acid, but a separation was not possible on account of the small quantity of material available.

SUMMARY.

The new mould metabolic product $C_8H_6O_6$ (M.P. 188–190°) formed from glucose by species in the *P. brevi-compactum* series, has been identified as 3:5-dihydroxyphthalic acid, and hence is constitutionally related to a large group of lichen acids.

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CCXXVII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXVI. THE FORMATION FROM GLUCOSE BY MEMBERS OF THE *PENICILLIUM CHRYSOGENUM* SERIES OF A PIGMENT, AN ALKALI-SOLUBLE PROTEIN AND PENICILLIN—THE ANTIBACTERIAL SUBSTANCE OF FLEMING.

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IN a recent paper, Fleming [1929] showed that a mould which appeared as an air contaminant in laboratory bacterial cultures, and which resembled *Penicillium rubrum* Grasberger-Stoll, produced when grown on nutrient broth a very powerful antibacterial substance. Filtrates of the broth cultures were named "penicillin" and it was found that the active substance affected different bacteria in different degrees. The action was very marked on the pyogenic cocci and the diphtheria group of bacilli, whilst the coli-typhoid and the influenza bacillus groups were quite insensitive to it. Staphylococci, *Streptococcus pyogenes* and the pneumococcus were completely inhibited in a 1 in 800 dilution of the filtrate, which did not affect the growth of *H. influenzae* (Pfeiffer's bacillus), and a method was described for the isolation of *H. influenzae* by incorporating penicillin in a suitable solid medium.

Examination of Fleming's organism by Dr Charles Thom at our request showed that it was not *Penicillium rubrum* but a strain closely related to *P. notatum* Westling in the *P. chrysogenum* Thom series. Dr Thom writes as follows: "I have cultivated Fleming's organism under several different conditions and cannot agree with the nomenclature as *P. rubrum* either in the sense of my 1910 paper or in the sense of Biourge's monograph. In fact, I believe this culture, although showing some divergencies in culture reactions, to be closer to *P. notatum* of p. 264 in my book than to the groups discovered on pp. 249–50 as indicated by the nomenclature used."

In his book [1930, pp. 261–2] Dr Thom says that the type strain of *P. chrysogenum* (No. 26) rapidly digests milk and liquefies gelatin giving a yellow colour. "Studied on a series of culture media, it changes colour with the composition of the substratum and with the age or stage of metabolism

of the colony itself: colonies grown on Czapek's solution in presence of abundance of fermentable sugar long remain green, without such sugar little or no yellow appears in the substratum." The production of this yellow pigment on milk appears to be characteristic of species in the *P. chrysogenum* series. One of the results of this work was the isolation and investigation of this pigment to which the name chrysogenin is given.

Although Fleming's organism appears, on the usual systematic criteria and from the fact that it produces chrysogenin, to belong to the *P. chrysogenum* series, the fact (see below) that we have never been able to demonstrate the production of the bactericidal substance, penicillin, by *P. chrysogenum* or by *P. notatum* renders the identification of Fleming's organism with either of these species doubtful from a physiological point of view.

Working both with Fleming's strain and the type strain of *P. chrysogenum* (No. 26), we have frequently experienced difficulty in repeating our experiments. Thus single spore cultures gave colonies sometimes with a white and sometimes with a yellow reverse, and when used for sowing flasks of our medium the former gave greater yields of chrysogenin than the latter. Moreover, with both strains after continued subcultivation on our medium, the mycelial felts tended to become less green, to show white patches, floccose in character, and this was always accompanied by decrease in the production of both chrysogenin and penicillin. From such cultures, by single spore technique, both floccose and green colonies were re-obtained, and the latter, but not the former, on using for sowing gave considerable chrysogenin formation (with all the strains) and penicillin formation (with Fleming's strain).

Eight cultures, for which we are indebted to our colleagues Miss L. Farrell and the late Mr J. H. V. Charles, have thus been employed.

Catalogue No.	Strain	Source
589	*P. chrysogenum* Thom (No. 26)	British National Collection of Type Cultures, Lister Institute
P 19 W	„ white reverse	Isolated from culture No. 589 at the London School of Hygiene and Tropical Medicine
P 19 Y	„ yellow reverse	Do.
P 19 Y_2	„ white floccose form	Do.
57	Fleming's strain (*a*)	British National Collection of Type Cultures, Lister Institute
152	„ (*b*)	Dr Fleming
I.D.	Fleming's strain, white floccose form	Isolated from culture No. 57 at the London School of Hygiene and Tropical Medicine
P 45	*P. notatum* Westling	Dr C. Thom

With all these strains (except the two giving a floccose type of mycelium, Nos. P 19 Y_2 and I.D.), the metabolism solutions gradually assumed a yellow colour and a negative rotation and gave a characteristic olive-brown colour with ferric chloride. The metabolism solutions on acidifying to p_H 3·5 gave a

yellow flocculent precipitate which was shown to consist chiefly of the yellow pigment chrysogenin, and a protein. Only the two green strains, cultures Nos. 57 and 152, and to a much smaller extent, the floccose culture I.D. from Fleming's culture, showed any antibacterial activity.

This activity, although arising more slowly on this synthetic medium, was as great or greater than that recorded by Fleming using a broth medium. This should be of considerable practical importance since the isolation of penicillin from a synthetic medium should be much simpler than from broth.

The purpose of the present paper is to describe the isolation of the pigment chrysogenin, and the protein, using the type strain of *P. chrysogenum* Thom No. 26, and the preliminary attempts at isolation of penicillin, using Fleming's culture.

Preparation of the three chief metabolic products.

A quantity of modified Czapek-Dox medium was made up having the following composition, for which we are indebted to our colleague Dr H. G. Turley:

$NaNO_3$	3·0 g.
KH_2PO_4	1·0 g.
KCl	0·5 g.
$MgSO_4, 7H_2O$	0·5 g.
$FeSO_4, 7H_2O$	0·01 g.
Glucose	40·0 g.
Distilled water to 1000 cc.	

400 cc. quantities of this medium were placed in 100 1-litre conical flasks which were plugged with cotton wool, and sterilised by steaming for half an hour on each of three consecutive days. Each flask was heavily sown with a suspension in the above medium of spores of a 6-day culture of either the type strain of *P. chrysogenum* or of Fleming's organism, grown on Czapek-Dox agar at 25°. The flasks were shaken and then incubated at 25° for 21–28 days, until the sugar had almost entirely disappeared, when a sample of the filtered metabolism medium from the type strain of *P. chrysogenum* gave in a 2 dm. tube a rotation of − 0·3 to − 0·5°. With Fleming's strain the rotation with optimum antibacterial activity was − 0·03 to − 0·08° but increased to − 0·36° during the subsequent few days.

The contents of the flasks were now filtered, the mycelium pressed and the combined filtrates (about 38 litres) were acidified to p_H 3·6 with about 480 cc. 2*N* H_2SO_4. With careful acidification a yellow precipitate was obtained which quickly flocculated, but if excess of acid was rapidly added the precipitate remained in an unfiltrable dispersed form. It was found with each organism that the crude precipitate consisted of about equal weights of protein and chrysogenin. In the case of Fleming's organism this precipitate could be filtered off almost without loss of antibacterial activity. A further small amount of chrysogenin can be precipitated from the filtrate by saturating

with salt and filtering. From a typical batch of 100 flasks (using the type strain of *P. chrysogenum*) 13 g. of crude precipitate were obtained containing 4·2 % of nitrogen by Kjeldahl. This material was then extracted exhaustively with ether in a Soxhlet apparatus and was thus separated into two fractions: (*a*) an ether-insoluble fraction containing 8·53 % N, (*b*) an ether-soluble pigment containing only traces of N.

Purification of the nitrogenous substance (protein).

The crude nitrogenous substance readily dissolved in dilute NaOH and could be roughly titrated by adding excess *N*/10 alkali and back-titrating using phenolphthalein as indicator. Thus 0·1903 g. of the crude material required 4·3 cc. *N*/10 NaOH. For purification, 7 g. of the substance were dissolved in excess dilute (160 cc. *N*/10) NaOH, diluted to 300 cc. and 200 cc. of absolute alcohol added. After filtering, 16 cc. of *N* HCl were added with vigorous shaking and the solution centrifuged. The supernatant highly pigmented layer was poured off. The process was repeated. The protein was then repeatedly shaken with 50 % alcohol (increasing gradually to absolute) and again centrifuged until all the colouring matter had been removed. The material was finally pressed on a Büchner funnel, washed with alcohol and ether and dried. The dried residue weighed 4·1 g., and had an ash content (Schoeller) of 0·54 %. The following figures were obtained by micro-combustion (Schoeller): C, 51·51, 51·76 %; H, 6·85, 6·95 %; N, 12·75, 12·95 %; S, 1·35, 1·32 %. A macro N determination also gave 12·95 % and this figure was used for the subsequent Van Slyke analysis of the protein.

0·3613 g. of this purified material required 3·6 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 1004.

Van Slyke analysis of the nitrogenous product (protein).

The above figures suggest that the substance is either a polypeptide or protein and since, so far as we are aware, no similar nitrogenous product has been isolated from mould metabolism solutions, a detailed study of the nature of the product was embarked on, by means of the Van Slyke nitrogen distribution method [1911; 1915].

Hydrolysis of nitrogenous product. 3·3439 g. of purified and dried product (containing 12·95 % N and 0·433 g. of total N) were boiled in a tared flask under reflux with 61·1620 g. of 20 % hydrochloric acid for 6 hours, the flask being heated in an oil-bath at 130–135°. The hydrolysis was then stopped, the flask cooled and its weight adjusted to the original weight by addition of water.

1 cc. of the fluid was then removed, made up to 10 cc. in a graduated flask and retained for the amino-nitrogen determination by the Van Slyke method. The remainder was re-weighed and the hydrolysis continued for a further period of 3 hours. The hydrolysis was then stopped, the weight adjusted, a

1 cc. sample removed and made up to 10 cc. for the amino-nitrogen determination as before, and the residue re-weighed.

Analysis of hydrolysis fluid. A small amount (2 cc.) of the diluted solution after 6 and 9 hours' hydrolysis gave 2·14 and 2·13 cc. amino-nitrogen respectively. It appeared therefore that the protein was readily hydrolysed, the hydrolysis being complete in 6 hours or less. In two other experiments the hydrolysis was almost complete in the same time using $2N$ H_2SO_4 instead of 20 % HCl. The amino-nitrogen figure (2·14 cc.) corresponds with a total amino-nitrogen of 0·3441 g., and the ratio after hydrolysis of amino/total nitrogen = 79·45 %. After removal of the samples for these determinations, the residue calculated on a weight basis corresponded with the presence of 3·224 g. hydrolysed protein or a total N content of 0·4176 g. This solution of hydrolysed protein was washed into a Claisen flask and evaporated to dryness *in vacuo* at 45°. Water was added and the evaporation continued, thus removing most of the hydrochloric acid. The residue was then dissolved in water and made up to 250 cc.

The total N was determined on two samples, each of 15 cc., by Kjeldahl and was 0·4176 g., agreeing exactly with the value obtained by calculation on a weight basis.

After removal of these samples, the residual solution corresponded to 2·837 g. of hydrolysed protein or a total N content of 0·3675 g., and the remaining determinations were made on this solution.

Ammonia was first determined in the hydrolysate by distillation with lime under reduced pressure. The melanin formed during hydrolysis and absorbed on the excess lime during the ammonia determination was filtered off and the nitrogen content obtained by Kjeldahl. The bases were then precipitated with purified phosphotungstic acid, filtered and washed, following the modified technique of Hartley [1914] and Van Slyke [1915]. The phosphotungstic acid precipitate of the bases was then decomposed by the hydrochloric acid-amyl alcohol-ether method [Van Slyke, 1915], the arginine being determined in the usual way, and the cystine by the Denis modification [1910] of the Benedict method.

Results. Ammonia. The amount of $N/10$ acid neutralised was 17·6 cc. indicating 0·0246 g. amide-nitrogen = 6·69 % of the total N.

Melanin. The amount of $N/10$ acid neutralised was 11·7 cc. indicating 0·0164 g. melanin-nitrogen = 4·46 % of the total N.

Cystine. The $BaSO_4$ weighed 0·043 g. indicating 0·0129 g. cystine-nitrogen = 3·51 % of the total N.

Arginine. The amount of $N/10$ acid neutralised was 5·8 cc. indicating 0·0324 g. arginine-nitrogen. Applying the cystine correction this reduces to 0·0302 g. arginine-nitrogen = 8·22 % of the total N.

Total nitrogen of bases. The amount of $N/10$ acid neutralised in the Kjeldahl determinations was 23·5 cc. Added to the amounts neutralised by the ammonia evolved in the arginine determination (5·8 cc.) the total acid

neutralised = 29·3 cc. indicating a total N of bases of 0·0820 g. N = 22·31 % of the total N.

Amino-nitrogen of bases. The mean amount of N gas evolved with 2 cc. (of 50 cc.) of solution was 2·73 cc. at 20° and 754 mm. indicating a total amino-nitrogen of bases of 0·0402 g. = 10·93 %.

Total nitrogen of filtrate from bases. The mean amount of $N/10$ acid neutralised per 25 cc. (of 150 cc.) was 29·5 cc. indicating 0·2478 g. N = 67·42 % of the total N.

Amino-nitrogen of filtrate from bases. The mean amount of nitrogen gas evolved with 2 cc. (of 150 cc.) was 5·3 cc. at 20° and 754 mm. indicating a total amino-nitrogen of filtrate of 0·2282 g. = 62·09 % of the total N.

The results are summarised in Table I.

Table I. *Results of protein analysis in percentages of total nitrogen.*

	Found g.	% of total nitrogen	Corrected for solubility of bases g.
Ammonia-N	0·0246	6·69	—
Melanin-N	0·0164	4·46	—
Cystine-N	0·0129	3·51	0·0155
Arginine-N	0·0302	8·22	0·0334
Histidine-N	0·0313	8·51	0·0351
Lysine-N	0·0076	2·07	0·0081
Amino-N of filtrate from bases	0·2282	62·09	0·2230
Non-amino-N of filtrate from bases	0·0196	5·33	0·0147
Total N	0·3708	100·88	—

The mould protein appears therefore to resemble fairly closely a typical alkali-soluble leaf protein. The following table gives the corresponding figures for the mould protein and the alkali-soluble protein of the alfalfa leaf [Dowell and Menaul, 1921].

Nitrogen content	Protein of alfalfa leaf %	Mould protein %
Total nitrogen	13·6	12·95
NH_3-N	6·8	6·69
Melanin-N	7·8	4·46
Arginine-N	11·01	8·22
Histidine-N	6·26	8·51
Cystine-N	0·85	3·51
Lysine-N	5·26	2·07
Monoamino-N	53·53	62·09
Non-amino-N	8·48	5·33

Action of trypsin and pepsin on the nitrogenous product.

The protein nature of the nitrogenous product was finally confirmed by its hydrolysis by trypsin in alkaline and by pepsin in acid medium.

A solution of 1 % protein in 0·5 % Na_2CO_3 was incubated at 37° after addition of trypsin along with controls containing on the one hand similar amounts of protein and carbonate, and on the other of trypsin and carbonate. 1 cc. of solution was removed for determination of amino-nitrogen by Van Slyke's method. The increase of amino-nitrogen (as cc. of nitrogen gas) per cc. of fluid due to the action of trypsin on the protein was from the blank value

of 0·38 cc. nitrogen to 1·08 cc. after 20 hours, and 1·40 cc. after 48 hours. A similar series of values was obtained from the action of pepsin in $N/30$ HCl, but the action was much slower. Thus the blank value of 0·43 cc. nitrogen increased to 0·75 cc. after 48 hours and to 1·05 cc. after 96 hours.

Isolation of chrysogenin.

The ether-soluble pigment chrysogenin, on removal of the ether was obtained as a transparent glaze on the side of the containing vessel. It was extremely soluble in most organic solvents except light petroleum in which it was insoluble. Attempts to crystallise it by slow evaporation of mixtures of solvents failed, the material always separating on the vessel as a glaze. It could, however, be precipitated from ether solution by light petroleum as a non-crystalline light yellow powder. This powder consisted chiefly of chrysogenin, together with a small amount of a reddish-brown dehydration product, the pigment readily losing water to form this compound. The dehydrated product was, however, not very soluble in benzene, and could be separated by shaking the yellow powder with benzene, filtering and reprecipitating the pigment with light petroleum. After repeating this treatment the product from several batches appeared homogeneous and gave the same analytical figures.

The following values were obtained on micro-combustion (Schoeller): C, 64·75, 65·06 %; H, 6·58, 6·51 %; OCH_3, nil. $C_{18}H_{22}O_6$ requires C, 64·69 %; H, 6·58 %.

This purified preparation was obtained in micro-crystalline form by dissolving in a very slight excess of $N/10$ NaOH and precipitating by passing a stream of CO_2. After precipitating in this way twice, filtering, washing and drying, a sample gave the following figures on micro-combustion (Schoeller): C, 64·82, 64·84 %; H, 6·52, 6·54 %; $C_{18}H_{22}O_6$ requires C, 64·69 %; H, 6·58 %.

The analytical figures agree very closely amongst others with those required by the formulae $C_{15}H_{18}O_5$, $C_{18}H_{22}O_6$. The molecular weight (Schoeller) was returned as 470, 490, but the results of titration and preliminary methylation experiments favour the formula $C_{18}H_{22}O_6$ (mol. wt., 334) and in view of possible errors in molecular weight determination by the Rast camphor method this latter value is provisionally adopted.

Properties of chrysogenin.

Chrysogenin is very soluble in ether, benzene, acetone, ethyl alcohol, ethyl acetate and chloroform, but insoluble in light petroleum and water. It gives a characteristic olive-brown colour with $FeCl_3$. It is very strongly *l*-rotatory, the specific rotation being $[\alpha]_{5461} - 762°$, $[\alpha]_{5790} - 570°$, and it is thus responsible for the development of the negative rotation in the metabolism medium. It possesses two very weakly acidic groups, and probably does not contain a free carboxyl group. Thus a solution containing 0·1320 g. of pigment, on

titration with $N/10$ NaOH to phenolphthalein, became more and more deeply reddish-brown until it was impossible to see the indicator colour, and the titration was continued using the indicator externally. After addition of 4·0 cc. $N/10$ NaOH (corresponding with an equivalent of 330, $C_{18}H_{22}O_6$ requires 334) the colour of the solution suddenly changed again from dark reddish-brown to light yellow. Further addition of NaOH led to a second increase of the red-brown colour and the end-point came after an addition of 8·22 cc. (corresponding to an equivalent of 160·6, $C_{18}H_{22}O_6$ assumed dibasic requires 167). On passing CO_2 through this neutralised solution the pigment again separated in microcrystalline form. Electrometric titration, kindly carried out by Dr Kekwick in Prof. Drummond's laboratory, showed that two stages of buffering were present, but the groups responsible were so weakly acidic that reliable dissociation constants could not be obtained, and only a rough value, 193, for the lower equivalent was given. The absence of a carboxyl group is also indicated by the fact that the substance does not esterify with ethereal diazomethane.

Chrysogenin on warming with dilute NaOH gives amongst other unidentified products acetaldehyde and sorbic acid. It does not methylate with ether-diazomethane, but gives with ether-nitrobenzene-diazomethane a monomethyl derivative which no longer gives the characteristic olive-brown colour with $FeCl_3$. The pigment reduces readily with Zn and HCl giving a colourless compound dihydrochrysogenin, $C_{18}H_{24}O_6$ (found: C 64·48 %; H 7·10 %; $C_{18}H_{24}O_6$ requires C 64·27 %; H 7·20 %) which readily re-oxidises in air, gives a wine-red colour with $FeCl_3$, and with ether-diazomethane gives a dimethyl derivative which shows a green colour with $FeCl_3$. The above facts suggest that chrysogenin contains a quinol nucleus. Investigation of its structure is reserved for a separate communication.

Antibacterial activity of the metabolism fluid of Fleming's organism.

A few preliminary experiments confirmed the observations of Fleming [1929] that *Bact. coli* and *H. influenzae* were resistant to the action of "penicillin" whilst *Staph. albus*, *Strept. haemolyticus*, *Strept. pneumoniae* and *N. meningitidis* were susceptible. The pneumococcus (*Strept. pneumoniae*) and Pfeiffer's influenza bacillus (*H. influenzae*) were selected for further experiments, the latter being used as a control.

Technique for determination of antibacterial power.

The metabolism fluid, after passing through a Seitz filter, was incorporated in both fluid and solid media.

Fluid media. Dilutions of the filtrate from 1/10 to 1/5120 falling in geometrical progression were made, using sterile precautions, in 1 cc. quantities of the test media. For the pneumococcus, Hartley's trypsinised horse muscle

broth was used, and for the influenza bacillus heart broth to which had been added 5 % of Fildes's extract [Fildes, 1920]. In each case a control tube was included containing the test media without the addition of metabolism solution.

After the preparation of the media, all the tubes were seeded with one loopful (approx. 1/250 cc.) of an 18 hours' broth culture of the test organism. All were then incubated at 37° for 24 hours and opacity readings taken. No difference between the early and late readings was apparent in a few experiments in which incubation was prolonged for 48 and 72 hours.

Solid media. In each experiment Petri dishes containing 15 cc. of solid media with the metabolism solution in a concentration of 1 in 20 were included, as a check on the detailed titrations in fluid media. The medium used for the pneumococcus was 5 % horse blood agar, and for the influenza bacillus 5 % Fildes's agar. Controls containing no metabolism solution were always included. The plates were streaked with one loopful of the test cultures.

Results of experiments.

The maximum yield of antibacterial substance produced by Fleming's culture in a broth medium appeared about the eighth day, which corresponds with the findings of Fleming. On the other hand, the yield in the synthetic medium was greater and reached its maximum concentration about the 16th to the 20th day. Further incubation resulted at first in a slight loss and later in a rapid loss of antibacterial activity. The following table gives the result of testing the yield over a short period.

Period of growth of Fleming's culture	Dilution of metabolism fluid inhibiting growth of	
	Strept. pneumoniae	*H. influenzae*
15 days	1/640	1/10
16 ,,	1/1280	1/10
18 ,,	1/640	—
22 ,,	1/640	1/10
24 ,,	1/160	1/10
31 ,,	1/160	1/10

In each experiment the control tubes gave a good growth of the test organism. On solid media, the pneumococcus was completely, and the influenza bacillus not at all inhibited at 1/20. It should be noted that this inhibition of *H. influenzae* in low dilutions occurred in relatively few experiments.

All subsequent work was carried out with filtrates from the growth of Fleming's culture on synthetic media.

Storage of filtrate.

Untreated filtrates taken when showing a high concentration of the antibacterial substance can be stored for quite long periods without great loss. A filtrate active against the pneumococcus in a dilution of 1/1280 was stored

in the ice-chest at 0° for 16 weeks without taking precautions as to sterility. Table II shows the reduction observed during that period.

Table II.

Period of storage in ice-chest at 0°	Dilution inhibiting growth of *Strept. pneumoniae*
Before storage	1 in 1280
After 5 days	1 in 1280
„ 7 „	1 in 640
„ 13 „	1 in 640
„ 14 „	1 in 640
„ 21 „	1 in 640
„ 12 weeks	1 in 320
„ 16 „	1 in 320

This method of storage was, however, inadequate as in two experiments the fluid became infected and antibacterial power was immediately lost. When the fluid was adjusted to p_H 5–6, passed through a sterilised Seitz filter into sterile flasks, and stored at 0°, the antibacterial power remained unchanged for 3 months. In all the experiments the p_H was readjusted to 7·2 before testing for antibacterial activity.

Antibacterial inactivity of chrysogenin and protein.

It has already been shown that careful adjustment of the p_H of the filtered metabolism solution to 3·6 caused the flocculation of most of the protein together with a large portion of chrysogenin. The filtrate, after removal of this precipitate and adjustment of the p_H to 7·2 still contained most of the antibacterial substance, whilst the precipitate on redissolving in dilute NaOH and readjusting the p_H showed no activity at all. Samples of dihydrochrysogenin, purified chrysogenin and protein also showed no activity.

Effect of evaporation of the metabolism fluid on the antibacterial power.

In order to deal successfully with the large bulk of fluid it was necessary to evaporate the fluid without serious loss of activity. Evaporation *in vacuo* at 40–45° of the filtered metabolism fluid to 1/20 its volume at p_H 2 and 3·5, with subsequent readjustment of volume and p_H resulted in complete loss of antibacterial power. Evaporation at p_H 7·2 also led to a fall of inhibitory power from a dilution of 1:1280 to 1:80. Between p_H 5 and 6, however, there appeared to be a region of greater stability and loss of activity was only small (1/640 to 1/320). It was, therefore, possible to adjust the filtered metabolism solution to p_H 3·6, to filter off most of the chrysogenin and protein, to readjust to p_H 5·5 and to evaporate *in vacuo* at this p_H, without great loss of activity.

Extraction of the antibacterial substance with ether.

Fleming regarded penicillin as insoluble in ether, but this was due to the fact that his filtrates were alkaline in reaction.

Extraction with ether at p_H 7·2 partly, and at p_H 2 completely, removes the antibacterial substance. Thus when the metabolism solution (150 cc.) was

extracted three times with ether (100 cc.) at p_H 7·2, the inhibitory power after removal of ether *in vacuo* at 40–45° fell from 1/1280 to 1/320, and after extraction at p_H 2 from 1/1280 to 1/10. When the ether of the ethereal extracts was removed in a current of air, the residue was inactive, but if the ethereal extracts together with 50 cc. water were evaporated *in vacuo* at 40–45°, the residual aqueous phase, after adjusting its volume and p_H showed a considerable portion of the original activity. Thus a solution originally inhibiting the growth of the pneumococcus in a dilution of 1/640 gave an extract showing inhibition at 1/160 (± 1/320).

From the above experiments it appears that the antibacterial substance is not identical with either chrysogenin or the protein, and that the greater part of these substances can be removed by suitable adjustment of p_H and filtering without serious loss of antibacterial activity. The antibacterial substance is extremely labile, becoming inactivated during evaporation of an ether solution in a stream of air and by evaporation *in vacuo* at 40–45° in both fairly acid and alkaline solutions. It is, however, more stable at p_H 5–6 and may be extracted with ether from acid solution. The investigation of the isolation and chemical nature of penicillin is being continued.

Summary.

The production, in synthetic media containing glucose and mineral salts with sodium nitrate as a source of nitrogen, of a new yellow pigment, chrysogenin, a new alkali-soluble protein and Fleming's antibacterial substance "penicillin" by several strains in the *P. chrysogenum* series is investigated.

Chrysogenin and the alkali-soluble protein are produced by normal strains of *P. chrysogenum* Thom, *P. notatum* Westling and by Fleming's organism. Neither of these substances is produced by the floccose forms of these organisms. Penicillin is produced only by Fleming's organism and not by the type strains of *P. chrysogenum* Thom and *P. notatum* Westling.

Chrysogenin, $[\alpha]_{5461} - 762°$, is responsible for the *l*-rotation and the olive-brown $FeCl_3$ reaction of the metabolism solution. It contains two very weakly acidic groups and is probably a quinol. The empirical formula $C_{18}H_{22}O_6$ is provisionally adopted. It does not show any antibacterial activity.

The protein is readily soluble in dilute alkali and a Van Slyke analysis shows that it closely resembles a typical alkali-soluble leaf protein. It does not show any antibacterial activity. This protein has thus been synthesised by the fungi from glucose as the sole source of carbon, and sodium nitrate as sole source of nitrogen.

Penicillin, previously obtained by growth of Fleming's species of *Penicillium* only in broth cultures, has now been prepared by growing this organism in a synthetic medium consisting solely of glucose and inorganic salts, thus permitting the hope of readier isolation. A technique for following the antibacterial power is described, and by its means the conditions for storage and

evaporation of active solutions without serious loss of activity, for separation of most of the pigment and protein and for extraction of the active material with ether, are investigated. Penicillin is an extremely labile substance, very readily inactivated by oxidation, evaporation *in vacuo* at 40–45° in both acid (p_H 2 and 3·5) and alkaline (p_H 8) solutions, but is moderately stable at p_H 5–6.

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CCXXVIII. THE BIOLOGICAL VALUES OF PROTEINS.

III. A FURTHER NOTE ON THE METHOD USED TO MEASURE THE NITROGENOUS EXCHANGE OF RATS.

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In the preceding papers of this series [Chick and Roscoe, 1930; Boas Fixsen, 1930] a description was given of the method used in this laboratory to estimate the biological values of proteins by the balance sheet method, using adult rats as experimental animals. Experience obtained subsequently in carrying out a large number of experiments has led to the introduction of certain modifications which seem worthy of note.

Technique.

In any experiment designed to investigate the value of a given protein for replacing the daily loss of body nitrogen, it is essential to secure for the experimental animal an adequate intake of calories derived from fat and carbohydrate in order to prevent the use of the food protein to provide fuel. Hence all experiments are discarded in which the daily intake of calories from fat and carbohydrate falls below 120 kg.-cal. (per 1000 g. body wt.) for rats below 375 g. in weight, and below 115 kg.-cal. for heavier rats. These figures have been arbitrarily selected after prolonged experience.

Every effort is made to secure a constant level of food intake during the experimental period of 4 days. The results of tests in which the weight of food consumed varied by more than 1 g. on any of the 4 days have not been used for calculations of the biological values of the proteins concerned.

During the periods when nitrogen-free diet is given in order to estimate the daily loss of endogenous nitrogen, it has not been found necessary to secure a large or uniform intake of food. No relationship has been found to exist between the output of endogenous nitrogen and the intake of food (see Table I). The probable explanation of this fact is that rats of the size used in this work (300–500 g.) have sufficient reserves of body fat and carbohydrate to supply their energy requirements during this period and need not draw upon their body protein, even when the food intake is small. The loss in body weight (av. 6 g. daily) was very small in comparison with the weight of the animal.

Table I. *Results of experiments in which N-free diet was given to adult rats.*

All figures are average daily amounts.

Rat No.	Date of exp.	Average body weight g.	Intake of dry food g.	Loss of body weight during exp. g.	Nitrogen in faeces mg.	Faecal nitrogen per 100 g. dry food mg.	Nitrogen in urine mg.	Total nitrogen output mg.
18	28. xi. 30	516	15·2	6	23·4	154·0	82·9	106·3
	15. i. 31	529	6·4	6	19·7	307·4	130·6	150·3
	12. ii. 31	537	10·6	6	26·9	253·5	111·9	138·9
	1. iv. 31	532	6·0	9	22·7	375·7	108·2	130·9
20	18. xii. 30	396	7·9	4	16·1	202·6	104·4	120·5
	12. ii. 31	433	10·5	5	20·0	189·9	111·1	131·1
	1. iv. 31	435	9·3	5	22·2	237·8	91·1	113·3
	14. v. 31	454	6·2	8	24·9	401·6	116·8	141·7
21	15. i. 31	310	7·6	2	21·2	280·4	96·3	107·5
	19. iii. 31	358	9·6	7	17·2	178·6	90·7	107·9
	30. iv. 31	383	2·7	7	16·0	592·6	80·9	96·9
	14. v. 31	375	2·4	9	12·7	529·1	98·4	111·1
23	19. ii. 31	374	8·1	8	15·0	185·2	63·5	78·6
	23. iv. 31	444	5·0	8	17·2	344·0	105·4	122·6
	18. vi. 31	488	4·5	8	18·2	404·5	81·8	100·0
	16. vii. 31	481	6·0	11	19·5	325·0	75·5	95·0
26	19. ii. 31	322	12·0	5	16·6	138·3	71·7	88·3
	30. iv. 31	347	11·3	4	15·7	138·9	77·2	92·9
	21. x. 31	396	11·1	5	20·3	182·9	81·6	101·9
	6. i. 32	416	5·4	7	15·6	288·8	88·9	104·5
	1. iii. 32	394	10·3	6	19·2	186·5	103·9	123·1
27	12. iii. 31	271	10·2	5	20·6	202·0	93·0	113·6
	7. v. 31	321	10·6	4	22·2	209·5	68·3	90·5
	24. vi. 31	339	6·0	7	22·1	368·3	97·7	119·8
	17. viii. 31	353	8·3	9	20·4	245·7	89·8	110·1
	30. ix. 31	358	5·3	9	16·5	311·3	100·8	114·5
	6. i. 32	388	0·8	8	16·2	2025·0	82·1	98·3
29	21. iv. 31	286	5·7	5	16·9	296·5	90·0	107·0
	16. vi. 31	329	3·9	10	11·9	305·1	92·9	104·8
	28. vii. 31	349	9·3	5	21·9	235·4	93·6	115·5
	29. ix. 31	386	1·8	2	13·3	739·0	99·3	112·6
31	13. viii. 31	357	8·7	9	21·6	248·3	86·9	108·5
	30. ix. 31	376	9·5	5	17·8	187·4	98·0	115·8
	6. i. 32	403	4·8	9	19·8	412·6	99·5	119·3
	2. iii. 32	388	8·2	7	22·5	274·5	91·9	114·3
	4. v. 32	406	9·7	6	16·9	174·3	79·9	96·8
	27. vii. 32	425	11·3	2	18·3	162·0	78·0	96·3
32	30. vii. 31	385	11·1	6	22·3	200·9	106·9	129·2
	28. ix. 31	356	12·3	5	18·5	150·4	75·4	93·9
	6. i. 32	401	5·6	5	15·6	278·6	78·3	93·9
	1. iii. 32	392	7·3	8	18·4	252·1	98·2	116·6
	4. v. 32	398	10·5	5	23·4	222·8	70·9	94·3
	8. vi. 32	409	8·2	6	18·4	224·4	86·4	104·8
	22. vi. 32	409	9·7	5	16·9	174·2	84·2	101·1
	6. vii. 32	396	8·0	6	14·7	183·7	80·8	95·5
35	11. xi. 31	243	5·2	7	15·1	290·4	66·0	81·1
	13. i. 32	291	5·2	5	11·2	215·4	79·3	90·5
	9. iii. 32	310	5·9	6	12·7	215·2	84·3	97·0
	11. v. 32	334	6·9	5	15·0	217·4	83·1	98·1
	13. vii. 32	381	9·1	4	18·5	203·4	79·7	98·2
37	11. xi. 31	294	8·8	5	18·9	214·8	81·9	100·8
	13. i. 32	339	7·2	5	17·5	243·1	107·8	125·3
	8. iii. 32	340	4·7	6	16·4	348·9	108·3	124·7
	11. v. 32	355	5·2	6	13·0	250·0	94·8	107·8
	8. vi. 32	374	6·9	5	18·8	272·5	88·3	107·1
	22. vi. 32	369	11·2	3	17·7	158·0	82·6	100·3
	6. vii. 32	368	8·7	7	14·0	161·0	77·1	91·1

Calculation of results.

The formula used to calculate the biological values of proteins

$$\left(100 \times \frac{\text{Body nitrogen saved}}{\text{Food nitrogen absorbed}}\right)$$

from the determinations of the nitrogenous exchange is derived from the graph given by Martin and Robison [1922], *viz.*

Biological value of protein $X = 100 \times (1 - \tan \theta)$, $\tan \theta$ being equivalent to

$$\frac{\text{Total N output in exp. with } X - \text{total N output on nitrogen-free diet}}{\text{True intake of N in exp. with } X}.$$

The nitrogen ingested during the nitrogen-free experiment is so small in amount, compared with the other values involved, that it can be disregarded.

The chief difficulties which arise in connection with obtaining a trustworthy estimate of the biological value of a protein by this method are the following.

(*a*) *The determination of the amount of faecal nitrogen of endogenous origin.* This figure is required for calculating all three terms in the above expression. In the first place, the total N output in the experiment with X is made up of the urinary nitrogen *plus* the endogenous faecal nitrogen. Secondly, the endogenous faecal nitrogen forms part of the total nitrogen output on the nitrogen-free diet. Thirdly, it is needed in order to calculate the amount of unabsorbed food nitrogen in the experiment with X (total faecal nitrogen — endogenous faecal nitrogen), which must be subtracted from the total nitrogen intake to get the true intake.

(*b*) *The proper interpretation of the variations occurring in the amount of total output of endogenous nitrogen,* at different times, with the same rat when receiving a nitrogen-free diet.

Determination of endogenous faecal nitrogen.

Mitchell [1924] argued that the amount of faecal nitrogen of endogenous origin excreted by a given animal was proportional to the weight of dry food ingested and that this ratio varied for different rats. In our experience however, the results of several series of experiments on the same rat receiving nitrogen-free diets (see Table I, col. 7) provide no evidence in support of such a relationship, at any rate where adult rats are concerned. On the contrary, in our work, the same rat has shown an excretion of faecal nitrogen which is roughly constant in amount, in spite of large variations in the food intake. Of 57 determinations made on 11 rats, 44 showed the endogenous faecal nitrogen excreted to be between 16 and 23 mg. daily. It was, decided, therefore, to take the actual amount of nitrogen excreted in the faeces of each rat during the period of nitrogen-free feeding as the measure of that particular animal's faecal output of endogenous nitrogen. Neither by this method nor by that used by Mitchell is it possible to make any allowance for the problematical influence on this amount of increased roughage or of a higher proportion of nitrogen in the diet. In any case, the value of the faecal nitrogen is so much smaller in amount than the other figures involved in the equation that

only very large variations in this figure would exert a significant influence on the final results.

Variation in total basal nitrogen expenditure.

In Mitchell's experiments young growing rats were used and the amount of nitrogen, per 100 g. body wt., excreted in the urine on a nitrogen-free diet was taken to be the measure of each animal's basal nitrogen metabolism, that excreted in the faeces being disregarded. In the present series in which the rats were full-grown, the quantity of nitrogen excreted in the urine was not found to be directly related to the body weight. Changes in the latter, as pointed out above [Boas Fixsen, 1930], usually indicated the deposition or removal of fat rather than alterations in the amount of active tissue. In some instances the endogenous urinary nitrogen remained stationary or became less as the rat became heavier (see rats 23, 31, 37, 35, Table I). Generally, the amount remained roughly constant and, of the 57 determinations given in the 8th column of Table I, in 47 the daily urinary nitrogen excretion lay between 78 and 120 mg. although the variations in body weight ranged from 271 to 537 g. As regards the range of variation in basal nitrogen expenditure shown in determinations made on the same rat at intervals of 6 to 8 weeks, Mitchell considered, on the basis of determinations carried out at shorter intervals, that these variations would be gradual and steady both in amount and direction, and that, therefore, the basal nitrogen expenditure at any period could be read off from curves in which the results of the different estimations had been plotted against time.

In the present investigation it was decided to take, as the estimate of daily nitrogenous expenditure for any particular rat, the average of all the determinations of total nitrogen output on a nitrogen-free diet which had been made on that animal within a period of 3 months before and 3 months after the experiment under consideration.

Summary.

Certain modifications are described in the method used to estimate the biological values of proteins from the results of measurements of the nitrogenous exchange. These are concerned with securing an adequate and uniform intake of calories and with obtaining a satisfactory estimate of the basal nitrogen metabolism.

We wish to express our gratitude to Dr H. Chick for her constant help and advice throughout this investigation. One of us (M. A. B. F.) is indebted to the Medical Research Council for a part time grant and to the Lister Institute for hospitality.

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CCXXIX. THE BIOLOGICAL VALUES OF PROTEINS.

IV. THE BIOLOGICAL VALUES OF THE PROTEINS OF WHEAT, MAIZE AND MILK.

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The relative efficiencies of the proteins of wheat, maize and milk in supplying the amino-acid requirements of the body have gained particular interest in connection with current theories of the etiology of pellagra [Wilson, 1921; 1930], a disease now generally accepted to be of dietetic origin. The majority of recorded outbreaks of pellagra have occurred among the poorer classes in districts where maize and maize products form the staple foodstuffs of the population and its occurrence among populations subsisting on wheat and rice, though recorded, is extremely rare. The disease, if not too far advanced, is cured after change to a "good diet" in hospital or elsewhere, and milk, meat and yeast have been shown to possess preventive and curative powers when added to a pellagra-producing diet [Goldberger, Waring and Tanner, 1923; Goldberger and Tanner, 1924; Tscherkes, 1930]. It is therefore reasonable to expect that any hypothesis as to the cause of the disease should account satisfactorily for these facts [see Aykroyd, 1930, 1].

Goldberger's vitamin theory [Goldberger and Tanner, 1925; Goldberger, Wheeler, Lillie and Rogers, 1926; Goldberger, 1927] attributes pellagra to a deficiency of the heat-stable vitamin B_2 component of the yeast vitamin B complex and would explain the curative action of the foodstuffs mentioned above on the ground of their high content of this vitamin. It does not, however, account for the association of pellagra with the consumption of maize, for Aykroyd and Roscoe [1929] have demonstrated that maize contains appreciable quantities of vitamin B_2, being in this respect in no wise inferior to wheat.

Turning to the theory elaborated by Wilson [1921; 1930] that pellagra is due to an amino-acid deficiency, *i.e.* to the ingestion of diets containing proteins of low biological value, the evidence is somewhat conflicting. Our present information as to the biological values of the mixed proteins of foodstuffs is derived from experimental work in which the three following methods have been employed.

(*a*) McCollum and Simmonds [1917], following a method used by Osborne and Mendel [1916] in their work on pure proteins, fed young rats on diets in which the foodstuff under investigation was the sole source of protein and determined the minimum percentage of protein in this form which must be given in the diet in order to prevent loss of weight. Using this method they concluded that the proteins of whole wheat and whole maize were of approximately equal nutritional value.

(*b*) In later work [McCollum, Simmonds and Parsons, 1921] the diets containing the foodstuffs to be investigated were so constituted that in each the proportion of protein was the same. The performances of rats fed on the different diets were then compared, not only as regards rate of growth, but also as regards longevity, reproduction and lactation, during, if possible, at least two generations. On the basis of experiments of this nature they were able to range the proteins investigated in the following descending order of merit as regards their biological value:

$$\text{Ox kidney} > \text{wheat} > \begin{Bmatrix}\text{milk}\\ \\ \text{ox liver}\end{Bmatrix} > \begin{Bmatrix}\text{ox muscle}\\ \text{barley}\\ \text{rye}\end{Bmatrix} > \begin{Bmatrix}\text{maize}\\ \\ \text{oats}\end{Bmatrix} > \begin{Bmatrix}\text{soya beans}\\ \text{navy beans}\\ \text{peas}\end{Bmatrix}$$

In this series the high value assigned to wheat and the low position of maize are noteworthy, and afford support to Wilson's theory of the aetiology of pellagra. Since, however, in these experiments the foodstuff tested was frequently the sole source not only of the protein, but also of the vitamin B complex, variations in the supply of one or other of the constituents of this complex might have influenced the results.

(*c*) The third method involves experiments of comparatively short duration and consists in determining the relative efficiency of different proteins for replacing the daily nitrogenous expenditure of the body—the "wear and tear" nitrogen—and for supplying the material for growth. The determination is made by the balance sheet method elaborated by Thomas [1909; 1910]. The figures obtained by Thomas in a heroic series of experiments on himself assigned the lowest value to the proteins of maize. The relative quantities of body nitrogen which could be replaced by 100 parts of the protein-nitrogen supplied from the following foodstuffs were as follows: from meat 109; milk 100; white wheat flour 40; maize 30. Thomas's figures were used by Wilson [1921] in his development of the theory that pellagra is the result of an amino-acid deficiency. It is obvious that calculations made on this basis would show diets containing a large proportion of maize to be markedly inferior in the biological value of their total proteins to those consisting largely of wheat, even if other deficiencies were present in the latter diets. Thomas's technique and conclusions have, however, been severely criticised from many points of view by Martin and Robison [1922].

McCollum [1911; 1914] also used the balance sheet method, working with pigs, and obtained results which, calculated by Thomas's formula [see

Chick, 1930] yielded the following estimates of biological value; milk, 74; caseinogen, 67; maize, 51; wheat, 45. A further series of experiments on the pig [Hart and McCollum, 1914] also showed the biological values of the proteins of wheat, maize and oats to be about equal. In these experiments the diets contained high percentages of protein.

The results obtained by Mitchell and his co-workers [Mitchell, 1924, 1, 2; Mitchell and Carman, 1924; 1926; Mitchell and Beadles, 1926] with young growing rats are also of interest. When the diet contained 8 to 10 % of protein, they obtained the following estimates of the biological value of the proteins of certain foodstuffs: whole milk, 85; liver or kidney, 77; ox muscle, 69; wheat, 67; maize, 60; white flour, 52. In these experiments the vitamin B complex was supplied as Harris yeast vitamin.

From the above facts it is apparent that the available data as to the relative nutritional values of the proteins of the different foodstuffs are extremely conflicting, whether the criterion employed in the tests has been the value for maintaining nitrogenous equilibrium in short-period tests or the value for supporting growth and development in long-period trials. The whole subject is reviewed in great detail in a critical article by Mitchell [1924, 3], to which reference should be made.

On the whole there is evidence for the superiority in nutritive value of animal protein over cereal protein, though McCollum and his co-workers found wheat proteins better than those of milk or ox muscle. They also found wheat superior to the other cereals tested including maize and in this advantage of wheat over maize they confirmed the result obtained by Thomas. But in the other researches cited, no significant difference has been demonstrated between the values of the two cereals.

The contradictory nature of these results suggested that a fresh assay of the biological values of some of the more representative foodstuffs, more especially wheat and maize, would be of interest, particularly as it is now possible to supply the constituents of the vitamin B complex in concentrates of relative purity, thus obviating some of the difficulties inherent in many of the estimations carried out previously [see Boas Fixsen, 1930].

Experimental.

Method.

The balance sheet method was used and the technique employed was that fully described in previous papers [Chick and Roscoe, 1930; Boas Fixsen, 1930] with the modifications given in the preceding paper [Boas Fixsen and Jackson, 1932].

Materials used.

Wheat products: whole wheat, white flour and wheat germ prepared from the same sample of soft red English wheat.

Maize products: whole maize and maize endosperm from the same sample of South American yellow corn.

Cow's milk: a preparation of whole winter milk dried by the roller process.

Caseinogen: "light white casein" (British Drug Houses), a sodium caseinogenate, manufactured by the method of Hammarsten.

Heated caseinogen: "light white casein" heated for 3 days at 112°, being stirred at regular intervals.

Lactalbumin: a sample prepared from whey.

"*Whole rat.*" After removal of the skin, six adult rat carcases were placed in boiling water for 10 minutes. The lungs, livers, hearts, kidneys and muscular tissue were minced, dried at 37° for 36 hours and finely ground. 1 g. of the product was the equivalent of 3·2 g. of the fresh material and contained 10·37 % of nitrogen.

Diets.

The nitrogen-free diet used had the following composition.

Corn starch	735 g.
Sugar	90
Clarified beef dripping	100
Cod-liver oil	20
Salt mixture	50
Calcium carbonate	8

The nitrogen content of different batches varied from 0·2 to 0·4 % of the dry weight and the calculated calorie value of the dry diet was 460 kg.-cal. per 100 g.[1]

In the experiments with "whole rat" these materials were administered by the method described in a previous communication in connection with heated caseinogen [Boas Fixsen, 1930]. The other substances tested were incorporated in diets so constituted that the ratio of fat to carbohydrate remained approximately the same. Table I gives the composition of two

Table I. *The constitution of two representative diets—WG and WF—in which the protein was derived from wheat germ and white flour respectively.*

The weights given refer to the air-dry materials.

		Diet WG g.	Diet WF g.
Wheat germ*, 220 g.	Protein	88	—
	Fat	26	—
	Carbohydrate	54	—
	Undetermined	52	—
White flour*, 600 g.	Protein	—	74
	Fat	—	8
	Carbohydrate	—	507
	Undetermined	—	11
Corn starch		600	226
Sugar		90	90
Clarified beef dripping		104	124
Cod-liver oil		20	20
Salt mixture		50	50
Calcium carbonate		8	8

* The figures for composition are taken from Osborne and Mendel [1919].

[1] The calorie value of this diet was previously wrongly given as 380 kg.-cal. per 100 g. [Boas Fixsen, 1930].

representative diets in which the protein was provided by wheat germ and wheat endosperm respectively, and Table II shows the estimated nitrogen and water contents and the calculated calorie values of the fat and carbohydrate of each of the diets used.

Table II. *Details of the diets used.*

Figures for water and nitrogen obtained by direct analysis.

Source of protein	Water content %	Nitrogen content of dry diet %	Protein content of dry diet (N × 6·25) %	Non-protein kg.-cal. per g. of dry diet
Whole wheat	10·10	0·890	5·6	4·1
White flour	10·32	1·125	7·0	4·2
Wheat germ	10·38	1·093	6·8	4·0
Whole maize	12·34	1·283	8·0	4·1
,,	8·00	0·781	4·9	4·4
Maize endosperm	9·50	1·072	6·7	4·6
Whole milk	8·66	1·097	6·9	4·4
Caseinogen	9·68	0·919	5·7	4·4
Heated caseinogen	9·02	1·110	6·9	4·5
Lactalbumin	9·54	1·074	6·7	4·5

Influence on biological value of the proportion of protein in the diet. Thomas [1909; 1910] assumed that, if sufficient fat and carbohydrate were supplied to cover the energy requirements of the organism, the capacity of any protein to replace the nitrogenous waste of the body tissue would remain constant at all levels of intake. Martin and Robison [1922] questioned the validity of this assumption on general grounds but found, nevertheless, in their own experiments made with the proteins of whole wheat, that the figure obtained for biological value remained the same when the diet contained the protein in widely different proportions. One of the present authors found the same to be true of heated purified caseinogen [Boas Fixsen, 1930]. Mitchell and other workers [Mitchell, 1924, 1; Morgan, 1931] on the other hand, found the biological values of many proteins to decline as the proportion in the diet was increased. In all the above tests the balance sheet method was used.

In the case of the proteins forming the subject of the present investigations, all those that have as yet been studied in different proportions in the diets have been found to show an increased biological value when given at the lower levels. This point is being made the subject of further study.

When the present investigation was planned it was not anticipated that the estimates of biological value would be influenced by the proportion of the protein in the diet. For this reason this level was not made uniform for all the proteins used. Whole wheat and caseinogen were studied in experiments so arranged that the proteins formed 6 % of the diet, with whole maize the levels of protein intake were 8 and 5 % and, with the other substances tested, 7 %. This has to be taken into account when deducing relative biological values from the results obtained.

Results.

The results of the experimental work are collected in Table III. The average biological values, given in the last column, agree fairly well with those of Mitchell.

Table III. *Details of the experiments in which the biological values of proteins in various foodstuffs were determined.*

The figures represent average daily values.

Source of protein used	% of protein in diet	Date of exp.	Rat no.	Body weight g.	Change in body weight g.	Intake of non-protein Calories per kg. body weight kg.-cal.	Intake of nitrogen (true) mg.	Output of nitrogen: Urine mg.	Output of nitrogen: Endogenous faecal mg.	Output of nitrogen: Total mg.	Biological value	Average
Whole wheat	6	29. iii.–2. iv. 31	21	379	+1	174	122·4	117·1	16·8	133·9	77	
		13–17. iv. 31	20	460	−3	124	98·6	138·9	20·8	159·7	67	
		,,	21	399	−2	138	98·6	121·7	16·8	138·5	67	
		27. vi.–1. vii. 31	23	503	−2	144	133·7	141·7	18·4	160·1	59	68
Wheat germ	7	16–20. xi. 31	26	420	−2	129	127·0	126·8	18·0	144·8	67	
		,,	31	406	−1	142	134·2	133·8	19·7	153·5	71	
		,,	32	416	−1	138	129·9	140·2	17·1	157·3	51	
		,,	27	408	−1	142	130·5	126·4	17·8	144·2	75	
		,,	34	401	−1	155	136·7	130·7	19·4	150·1	83	69
Wheat endosperm	7	11–15. v. 31	26	362	−3	136	123·6	123·7	16·2	139·9	60	
		27. iv.–1. v. 31	20	460	−2	117	129·9	159·3	19·4	178·7	56	
		4–8. v. 31	23	454	+1	139	151·0	145·7	17·5	163·2	57	
		20–24. iv. 31	27	321	+2	192	155·9	133·1	21·8	154·9	69	
		,,	28	328	−1	154	123·1	120·5	17·6	138·1	62	61
Whole maize	8	17–24. iii. 31	12, 15	393	+1	185	195·6	147·0	22·9	169·9	63	
		,,	11, 16	383	+2	188	177·5	138·6	15·7	154·3	71	
		17–21. iii. 31	13, 14	375	+1	142	144·9	108·9	32·9	141·8	68	67
	5	26. iv.–1. v. 31	11, 15	418	−2	139	79·6	97·0	19·5	116·5	85	
		,,	12, 16	416	−2	160	99·5	96·2	20·3	116·5	78	
		,,	13, 14	392	−1	151	97·3	92·2	22·9	115·1	81	
		29. xii. 30–2. i. 31	20	414	−3	119	76·5	114·1	18·1	132·2	92	84
Maize endosperm	7	9–13. ii. 31	21	371	−2	187	135·0	134·9	16·8	151·7	66	
		,,	22	389	−4	173	135·4	136·0	19·5	155·5	71	
		16–20. iii. 31	18	546	−2	133	150·8	142·6	23·2	165·8	77	
		,,	20	441	−3	123	122·4	149·3	20·8	170·1	65	70
Whole milk	7	20–24. iii. 32	37	342	+1	136	103·4	111·3	15·6	126·9	93	
		,,	41	305	−1	172	119·1	84·3	26·1	110·4	88	
		,,	35, 36	345	−1	129	89·3	104·6	13·3	117·9	77	
		4–8. iv. 32	31	408	−1	136	118·3	105·7	19·7	125·4	87	86
Lactal-bumin	7	1–5. ii. 32	42	308	−4	126	83·1	108·6	11·3	119·9	70	
		,,	26, 31	406	−3	131	121·8	137·3	19·2	156·5	66	
		,,	32	416	−2	139	128·7	135·7	17·0	152·7	63	
		8–12. ii. 32	36	345	−4	147	106·6	130·6	14·4	145·0	60	65
Caseinogen	6	23–27. xi. 31	35	262	+2	169	94·0	78·0	13·2	91·2	94	
		,,	37	403	−1	144	118·0	124·2	18·3	142·5	75	
		,,	39	388	−3	116	87·9	95·6	19·9	115·5	79	
		10–14. i. 32	38	337	−1	133	91·9	100·1	7·5	107·6	81	
		25–29. i. 32	39	316	−6	157	106·9	125·4	18·2	143·6	57	
		,,	37	344	−1	156	114·1	127·5	17·6	145·1	75	
		,,	35	300	−1	159	94·4	101·6	13·0	114·6	74	76
Heated caseinogen	7	22–26. ii. 32	41	299	+1	167	115·6	119·3	26·1	145·4	57	
		,,	43	361	−4	146	121·2	169·0	18·4	187·4	49	
		28. ii.–3. iii. 32	42	297	−2	180	117·9	135·0	11·3	146·3	57	54
"Whole rat"	7	27. vi.–1. vii. 32	31	431	−1	>122	139·7	157·3	17·6	174·9	44	
		,,	35	377	0	>142	143·0	132·9	16·8	149·7	64	
		,,	41	355	−1	>145	134·9	135·4	22·8	158·2	53	
		,,	43	424	−1	>130	140·0	149·0	19·5	168·5	57	
		25–29. vii. 32	41	361	−1	>141	145·1	137·2	20·7	157·9	57	55

Seeing that the figures obtained from different experiments with the same protein showed variations of from 10 to 20 %, only large differences between the average figures obtained for the different proteins are considered to be of significance. When this is allowed for, the following conclusions can be drawn.

Wheat and maize proteins. There is little significant difference between the values found for whole maize (67 and 84 at 8 and 5 % levels, respectively) and whole wheat (68 at a 6 % level). Wheat germ gave a value of 69 (7 % level) and wheat endosperm 61 (7 % level) which is in accord with the observation of Klein *et al.* [1926] that in wheat the proteins of the germ are superior to those of the endosperm.

Milk protein. The proteins of whole milk were found to possess the highest value of the series tested, with an average figure of 86 when given in a diet containing 7 % protein. Caseinogen and lactalbumin were both inferior, giving figures of 75 and 65 for protein intakes of 6 % and 7 % respectively, suggesting that these two proteins exert a supplementary action upon one another in their mixture in milk. The slight superiority of caseinogen over lactalbumin is contrary to what would be expected from the available data concerning the amino-acid make-up of these two proteins, seeing that analysis of the latter shows a larger proportion of the important amino-acids tryptophan, cystine and lysine. Osborne and Mendel [1916] found lactalbumin to be superior to caseinogen for both growth and maintenance of young rats.

Purified caseinogen (extracted with acidified water and with dilute alcohol and then heated for 3 days at 115°) when evaluated previously [Boas Fixsen, 1930], was found to have an average biological value of 45 (50 when calculated by the modified method now used) at all levels. The discrepancy between this figure and the higher values obtained by other workers [McCollum, 1914; Mitchell, 1924, 2] was at that time tentatively ascribed either to differences in technique, or to the removal during purification of traces of a contaminating protein with a biological value higher than that of caseinogen. Morgan's work [1931] has shown, however, that it should be attributed to the marked deterioration in biological value suffered by a protein as the result of prolonged heating at a relatively high temperature. This is confirmed in the present work, in which unpurified caseinogen was evaluated at 76 (6 % level), while caseinogen which had been heated but not extracted had sunk in value to 54 (7 % level).

The low value of 55 given by the "whole rat" preparation is of interest. Theoretically one would expect the proteins of the rat to have for that animal a value approaching 100, by analogy with Michaud's [1909] experiments in which dog's flesh was found to have an efficiency of 100 % in replacing the dog's daily nitrogen excretion.

Applicability of the experimental results to the current theories of the aetiology of pellagra.

It is difficult to reconcile the above observations on wheat and maize with the theory that pellagra is caused by an inferiority in the quality of the protein

consumed on the maize diets, *i.e.* to an amino-acid deficiency. Since 58 % of the protein of maize [Osborne and Mendel, 1913] is the notoriously incomplete zein, one must conclude that maize glutelin, which constitutes 36 % of the total protein, forms an effective supplement to zein in maintaining nitrogenous equilibrium in the adult animal. Osborne and Mendel [1914], indeed, found maize glutelin to possess a high nutritive value.

On the other hand it is not easy to decide how far results of the kind described in this paper obtained in short term experiments on rats are applicable to the case of human beings subsisting for long periods on a diet consisting largely of maize or to what extent they are of importance in the aetiology of pellagra, a human disease developing after many months' subsistence on a faulty dietary regime.

It is worth noting, nevertheless, that six days (the duration of each metabolism experiment including the preliminary period) in a rat's life is roughly equivalent to 4 to 5 months of a man's life. In Goldberger's Rankin farm experiment [Goldberger and Wheeler, 1920] the first symptoms of pellagra appeared after 5 months' subsistence on the experimental diet.

The balance sheet method as used by us is concerned only with the relative efficiency of proteins in replacing the daily nitrogenous expenditure of the body, *i.e.* with the function of maintenance. In McCollum's long-term experiments, on the other hand, growth, reproduction and lactation were also involved. It is probable that for these functions a disparity exists between proteins shown to be of equal value as regards maintenance[1]. The inferiority of maize to wheat, when given as sole source of protein in diets employed for rearing young rats to maturity, has received some measure of confirmation in recent experiments in this laboratory. Pellagra, however, as was pointed out by Mitchell [1924, 3] in this connection, is a disease mainly of adult life and not specifically associated with periods of growth or adolescence.

In considering the theory linking pellagra with a simple amino-acid deficiency, a fact to be noted is the low calorie value of all the pellagrous diets quoted by Wilson, except that used in Goldberger's Rankin farm experiment. In consequence, much of the already low protein intake must have been used for fuel, so that whatever the biological value of the protein when used solely to supply nitrogen, a deficiency of protein would inevitably result. In every case cited where pellagra was cured by the addition to the diet of animal protein, the calorie value was at the same time raised, thus automatically improving the value of what was previously the sole source of protein. Many of the non-pellagrous diets were also of low calorie value, so that in these also the biological value of the protein must have been much lower than that calculated by Wilson, even if Thomas's figures from which the calculations were made are considered trustworthy.

[1] It is interesting to note in this connection that in South Carolina mill villages in 1917 the incidence of pellagra per 1000 persons from 20–29 years of age was 61 females to 3 males, while the ratio of married pellagrins to those found among single women was 52 to 12 [Smith, 1931].

It is evident, therefore, that the experimental support for the theory connecting pellagra with a simple amino-acid deficiency in the diet, is not as strong as has been supposed. The superiority in biological value shown by proteins of animal origin is in accord with the proved curative value of animal foods, but the fact that maize has not been found consistently inferior in this respect to wheat or certain other cereals fails to account for the known association of the disease with the consumption of maize. In this respect, therefore, the theory relating pellagra directly to a deficiency of protein of high biological value has not been more fortunate in its experimental support than the theory of vitamin deficiency put forward by Goldberger and his associates (see above).

It must be admitted therefore that as yet there is no satisfactory explanation of the fact, well established in human experience, that maize possesses a nutritive value inferior to that of wheat and that there is a greater liability to nutritional disease on diets consisting largely of the former cereal. Maize is eaten of necessity and never of choice if wheat is available, which fact probably provides an additional proof of its inferiority. The theory has frequently been advanced that a toxic substance is present in certain portions of the maize grain or may be developed under certain conditions. There is, however, very little direct evidence in its support. The oft-quoted experimental work of Horbaczewski [1910; 1912] was interpreted to show that the skin lesions of pellagra were due to an increased sensitiveness to strong sunlight caused by absorption of this toxin. Although his experiments lack confirmation [Aykroyd, 1930, 2] the widespread suspicion that some definitely unwholesome substance may be present in maize deserves further investigation.

Summary.

1. The following figures have been obtained for the biological values of the proteins of certain foodstuffs, using the balance sheet method with adult rats as the subjects, biological value being defined as the number of parts of body nitrogen replaced by 100 parts of the protein investigated,

$$\text{or} = 100 \times \frac{\text{Body N saved}}{\text{Food N absorbed}}.$$

Whole wheat (6 % protein in diet) 68; wheat embryo (7 %) 69; wheat endosperm (7 %) 61.

Whole yellow maize (8 %) 67; (5 %) 84; yellow maize endosperm (7 %) 70.

Whole milk (7 %) 86; lactalbumin (7 %) 65; caseinogen (6 %) 76; heated caseinogen (7 %) 54·5.

"Whole rat" preparation (7 %) 55.

2. From these figures certain conclusions have been drawn.

(*a*) No significant difference is shown between the biological values, as defined above, of the proteins of wheat and maize.

(*b*) The proteins of the embryo of wheat are of slightly superior value to those of the endosperm.

(*c*) Caseinogen suffers a marked decline in biological value as the result of prolonged heating at 112°.

(*d*) Caseinogen is a slightly more valuable protein for maintenance than is lactalbumin.

(*e*) Caseinogen and lactalbumin appear to have a supplementary action towards one another, the proteins of whole milk being superior in value to either protein ingested separately.

3. The experimental study of the biological value of maize and wheat proteins lends little support to the theory that pellagra among maize eaters is the result of a simple amino-acid deficiency. So far no satisfactory explanation is forthcoming of the general experience of mankind that diets consisting largely of maize have been found of inferior nutritive value to these containing wheat as the principal foodstuff.

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CCXXX. THE CHEMISTRY OF MOULD TISSUE[1]. I. SOLUBLE CARBOHYDRATE CONSTITUENTS.

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No very detailed chemical examination of the carbohydrate constituents of any fungal material has ever been made, but numerous references exist to the isolation of individual polysaccharides from such tissue. In some cases the names which have been applied, *e.g.* fungal starch, cellulose, *etc.* are misleading, inasmuch as they suggest an identity with these plant constituents. The chemistry of fungal tissue has an individuality of its own and it is probably true to say that, while it differs in numerous respects from that of both plant and animal tissues, it more nearly approaches that of the latter than of the former. Protein and nitrogenous material are quantitatively more important than the carbohydrates in most cases, and frequently also the fat and combined fatty acid contents are quite high—conditions very different from those obtaining in mature plant tissues.

In the closing years of the last century much work was carried out on fungal mycelium from a histological point of view. Reliance was placed mainly on so-called selective staining reactions in combination with certain extraction procedures. Most of the workers concerned were impressed by the complex and resistant character of the tissue, and explained the difficulties they experienced in extraction on the basis of infiltration of one constituent into another and the presence of insoluble combinations between such constituents as fatty acids and proteins, cellulose and nitrogenous substances.

A number of carbohydrate preparations have been obtained from fungal material. Cramer [1894] isolated from the spores of *Penicillium glaucum* a polysaccharide giving a blue colour with iodine. This he termed spore starch, and showed that it could be completely hydrolysed in 3 hours by boiling with 1·25 % sulphuric acid. Mangin [1890] reported and studied a carbohydrate, callose, which he isolated from the hyphae of many species of fungi. He found it to be insoluble in water and alcohol, but easily soluble in cold alkali, and described a number of staining reactions for it, some of which are of questionable specificity. Winterstein [1894] obtained from *Boletus* by acid extrac-

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tion a carbohydrate which he termed paradextran and later from *Polyporus* a similar material, para-isodextran. The latter he showed to yield glucose on hydrolysis, to give an intense blue colour with iodine, and to have $[\alpha]_D = +240°$ in solution in 5 % alkali. Since these preparations were apparently soluble in alkali, it is probable that they represent the same fraction as that obtained by Mangin and described as callose.

Dox and Neidig [1914, 1] prepared polysaccharides from the hot water extract of various fungi. From *Penicillium expansum* they obtained material, insoluble in cold water, termed by them mycodextran, highly dextrorotatory and yielding glucose only on hydrolysis. It was unattacked by enzymes and gave no coloration with iodine when pure. Later [1914, 2] they described a similar compound from *Aspergillus niger*, stated to give only galactose on hydrolysis. They express the opinion that both these polysaccharides are reserve substances to be used by the fungi when the carbohydrates in the medium are exhausted, since they were unable to obtain any from mature or partly autolysed cultures.

Boas [1917] investigated the cultural conditions necessary for the production of fungal "starch," but attempted no chemical work on this substance, relying on iodine coloration as an index of its presence. Schmidt [1925] considered the production of fungal "starch" in *Aspergillus niger* to be dependent on the nitrogen-carbohydrate ratio of the medium, a low ratio favouring "starch" production. The "starch," which was water-soluble, was hydrolysed by various enzymes and by acids. She believed it to be identical with amylose, and *iso*lichenin, but it must be pointed out that there is no quantitative evidence in support of this view. Takata [1929] isolated from *Aspergillus oryzae* a water-soluble preparation, said to be identical with that of Schmidt, and yielding glucose and a little mannose on hydrolysis either with acid or takadiastase. He also obtained an alkali-soluble polysaccharide by extraction with 10 % sodium hydroxide. This preparation had a nitrogen content of 4·4 % and was hydrolysed to reducing sugars only with difficulty. An appreciable amount of glucosamine was given on hydrolysis.

Raistrick and co-workers obtained a number of carbohydrates from fungi. Birkinshaw, Charles and Raistrick [1931] prepared from various species of *Penicillium digitatum* a hot water-soluble polysaccharide which separated out on cooling. This probably corresponds to the mycodextran of Dox and Neidig [1914, 1] since it yielded only glucose on hydrolysis, was highly dextrorotatory, gave no colour with iodine and was unaffected by diastatic enzymes. From *Fumago vagans*, Birkinshaw and Raistrick [1931] isolated a water-soluble carbohydrate, precipitable by alcohol, also yielding only glucose on hydrolysis and unaffected by enzymes.

Most of the investigations mentioned above deal with one isolated constituent of the mould tissue, and few attempts have been made to examine the tissue as a whole. Perhaps the only recent survey which has been made is due to Thomas [1928] working with the hyphae of *Fusarium*, and later [1930] also

with *Sclerotium*. Like the earlier workers he too relied extensively on staining reactions and colorimetric tests and few analytical data are given. He points out that the apparent resistance of the hyphae towards most reagents is due to the presence of fatty substances and fatty acids in the cell-wall and postulates cellulose-fatty acid, and protein-pectin complexes incrusting and impregnating a basic skeleton of a chitinous nature.

This review would seem to indicate that different species show considerable variation in the nature of the carbohydrate material present. The majority of these preparations are, however, small fractions easily soluble, and do not represent the larger structural components which may, as in the higher plants, exhibit more constancy.

Experimental.

(*a*) *Material employed.*

In the work to be described, the fungal tissue investigated was from one strain of *Aspergillus fischeri* only. It was obtained from Dr Charles Thom of the U.S. Department of Agriculture, and was known by him as No. 5041 of his collection. It develops on liquid media in the form of a thick mycelial felt or pad, and by cultivation in pans in a specially designed incubator of the type described by Raistrick *et al.* [1931] and Peterson *et al.* [1932] may be obtained in quantities sufficient for extensive chemical investigation. The medium throughout was glucose-ammonium nitrate[1], initially at a p_H of 6·8 and the conditions of fermentation were those outlined by Peterson *et al.*

When harvested, the mycelium was usually not very heavily sporulated, though considerable variation in this and in the mycelial weight from a given concentration of sugar was observed. In general the tissue taken was of the same age, but it was evident that it was not always of equal maturity. Since there is no rational criterion of maturity, it was not possible to examine the effects of such differences.

The tissue was available in four different conditions: (1) fresh, (2) killed by heating to 99°, (3) killed at 99° and dried at 67°, (4) killed at 99°, dried at 67° and extracted for many hours with alcohol at 80°. Of these, the fresh material is much to be preferred. The process of killing by heat followed by drying or treatment with alcohol seems to produce changes in physical properties both of the protein and carbohydrate constituents, which render subsequent handling more difficult and give preparations of a poor colour. The medium employed for the growth of the mould had a high content of inorganic salts and despite careful washing of the mycelial felt, salts (mainly phosphate, magnesium and sulphate ions) gave considerable trouble later in the preparations. The underside of the pad was of a firm gelatinous nature and appeared to retain these physically.

[1] The composition of the medium was: cerelose (glucose), 100 g.; NH_4NO_3, 10 g.; KH_2PO_4, 6·8 g.; $MgSO_4,7H_2O$, 5·0 g.; *M*/1 $FeCl_3$, 0·5 cc.; 1 % $ZnSO_4,6H_2O$ sol., 5·0 cc.; water, 1000 cc.

(*b*) *Distribution of constituents.*

A typical sample of dried tissue gave on analysis C, 43·8 %; N, 5·4 %; ash, 5·67 %. The nitrogen content varied with different samples, but was over 5 % in every case, the highest figure obtained being 6·02 %.

A preliminary survey of the distribution of constituents was made by determining the loss on treatment with various reagents. This indicated that the carbohydrate material of the tissue may be placed in three groups: (1) water-soluble, (2) alkali-soluble, (3) alkali-resistant residue. Protein was present in both watery and alkaline extracts along with the carbohydrate.

Typical figures are given in Table I for the watery and subsequent alkaline extracts of fresh living tissue (A), heat-killed and dried tissue (B) and heat-killed and alcohol-extracted tissue (C).

Table I.

Extract		% removed	N content of extract g.	N calc. as protein g.	Probable carbohydrate by difference %
Hot water	(A)	37·9	3·10	19·4	18·5
2 extractions	(B)	25·5	1·74	10·9	14·6
	(C)	12·8	0·59	3·7	9·1
Hot 2 % alkali	(A)	32·2	1·96	12·3	19·9*
3 extractions	(B)	36·2	3·14	19·6	16·6*
	(C)	43·2	4·30	26·9	16·3*

* Possible saponification of fats by this treatment has not been considered.

It will be seen that the amount of material extractable by these two processes was influenced to a marked degree by the condition of the tissue. The non-nitrogenous material, presumably carbohydrate, removable by water and alkali respectively was approximately equal in amount from the fresh and the dried tissue. In view of the higher percentage removal from fresh tissue, this was employed in practically all the preparations to be described.

(*c*) *Sugars present.*

About 2 g. of the dried tissue were hydrolysed by boiling for 5 hours with 3 % H_2SO_4. The filtrate was neutralised with barium carbonate, decolorised with norite and concentrated under reduced pressure to a small volume. On preparation of the osazone, glucosazone only was obtained. An attempt to obtain a hydrazone in the cold (mannose) was unsuccessful. The glucosazone was recrystallised from pyridine and the melting-point found to be identical with that of glucosazone (M.P. 204°).

A mucic acid test for galactose by oxidation of the tissue with nitric acid gave negative results. A uronic acid determination by distillation with 12 % HCl yielded only traces of CO_2 and indicated the absence of this group.

Furfuraldehyde estimations for pentoses were carried out on several samples and a trace only was found to be present. Care has to be exercised in carrying out this estimation on mould tissue since some fatty acid distils over with the HCl-furfuraldehyde and the distillate must be filtered before the

addition of phloroglucinol. The precipitated phloroglucide was extracted with hot alcohol in the usual manner to remove the phloroglucide of ω-hydroxymethylfurfuraldehyde due to hexose units.

Furfuraldehyde from alcohol-extracted tissue, 0·56 % = anhydropentose 0·96 %.

Furfuraldehyde from fresh tissue grown on 5 % xylose, 0·39 % = anhydropentose 0·67 %.

The nucleic acid of *Aspergillus oryzae* has been shown [Takata, 1929] to contain pentose, indeed pentose forms an appreciable percentage of the molecule. It would not be unreasonable to suppose therefore that this small quantity of pentose found in the tissue might be due to nucleic acid. Even with xylose as the sole source of carbon, the tissue appeared to contain only glucose units in its polysaccharides.

(*d*) *Water-soluble carbohydrate.*

(1) *Preparation and properties of the acid-alcohol precipitate* (Fraction A). When fresh material was used it was thoroughly washed with cold water, and ground to a pulp in a mill before extraction. Very satisfactory grinding was effected if the tissue was first frozen. When dried material was employed, this was ground to a fine powder and allowed to soak for several hours in order to get satisfactory penetration. Two extractions were then made with hot but not boiling water, and filtration was effected first on a cloth and then through paper. A golden yellow solution was obtained, filtering rapidly while hot, but only slowly when cold. No fraction comparable with the mycodextran of Dox and Neidig [1914, 1] separated out on cooling. The solution was concentrated under reduced pressure, again filtered and poured into 2·5 volumes of alcohol containing a little HCl. A white flocculent precipitate appeared, settling readily. The filtrate was retained. The precipitate was washed by repeated sedimentation in acid-alcohol and finally dried in alcohol of increasing concentration. It will be referred to as Fraction A. The importance of adequate washing cannot be overemphasised, for unless this was thorough, the finished preparation contained much ash. The quantity of the fraction precipitated by acid-alcohol seems to vary considerably with the material; in one or two cases practically none of this fraction was obtained. It dried easily to a white powder, soluble in water with some difficulty. Unlike the water-soluble fungal "starch" obtained by Schmidt [1925], by Boas [1917] and by Cramer [1894], it gave no coloration with iodine. Though the major part of the protein was no doubt coagulated by the heating process, this fraction usually contained a little nitrogen, presumably in the form of protein. The preparations were hydrolysed readily with dilute acid to give reducing sugar, characterised by means of its osazone as glucose. No hydrazone was obtained in the cold, indicating the absence of mannose. The mucic acid test for galactose and naphthoresorcinol test for uronic acids were both negative.

The dried preparations redissolved in water only with difficulty and gave

a somewhat dark solution. The rotation of one preparation was found to be $[\alpha]_D^{22°} = 86{\cdot}2°$.

When in solution this carbohydrate passed slowly through a cellophane membrane (Du Pont—600). The integrity of the membrane was subsequently verified by dialysing starch against a dilute iodine solution. The starch was retained.

The rates of hydrolysis of two typical preparations are given in Table II (sample I, ash 3·5 %; N, 1·33 %; sample II, ash 1·3 %; N, 0·87 %).

Table II.

% reducing sugar calc. as glucose.

Hours ...	½	1	1½	2	3	4	16	18	24
Sample I	18·2	46·5	58·3	59·5	64·7	67·4	—	—	80·3
Sample II	31·9	45·5	—	62·5	79·4	84·9	94·8	80·5	—

To obtain confirmatory evidence as to the nature of the sugar, rotations were determined after hydrolysis. 0·197 g. in 25 cc. 3 % H_2SO_4 was boiled for 5 hours, neutralised with barium hydroxide, boiled with a little norite, filtered and evaporated down to less than 5 cc. *in vacuo*. After making up to 5 cc. the rotation was observed in a 2 dm. tube as + 2·41°. The sugar concentration was then determined and found to be 0·0232 g. per cc. calculated as glucose. On this basis $[\alpha]_D = \frac{2{\cdot}41}{{\cdot}0232 \times 2} = + 51{\cdot}9°$. In view of the close approximation to the theoretical value for glucose, this observation provides a further indication that the main if not the only sugar to be obtained on hydrolysis of this carbohydrate is glucose.

(2) *Other water-soluble carbohydrate fractions.* An investigation of the alcoholic filtrate from the precipitation of the acid-alcohol fraction (Fraction A) showed that more carbohydrate material remained in solution and could not be precipitated by increasing the alcohol concentration. Two crude fractions were obtained, one by making the solution alkaline, and the other by the subsequent addition of lead acetate in acetic acid. The nitrogen content of these precipitates was low, and some glucose was obtained on hydrolysis. These fractions have not yet been further investigated.

(*e*) *Alkali-soluble carbohydrates.*

(1) *Preparation of crude material.* As shown earlier, an alkaline extract of mould tissue contains in addition to carbohydrate material considerable quantities of protein, possessing very similar properties in so far as precipitability is concerned. Preparations obtained by methods similar to those employed for the alkali-soluble constituents of plant material (hemicelluloses) have, in consequence, a high protein content and are of little value in work on the polysaccharides. The procedure generally followed in making an extract was to treat the residue first with 2 % NaOH in the cold, and then again with the same concentration of alkali slightly warmed. In some cases a treatment with hot 2 or 4 % NaOH followed, but in general this was omitted since the

object was not so much to obtain complete extraction of the tissue as to get a preparation of high purity. The extracts were obtained by filtration through muslin or cheese cloth and the residues pressed out. Particles of tissue were removed by sedimentation and filtration through a thick pad composed of glass-wool and cotton. To the dark brown liquid obtained was added a definite excess of HCl so that the mixture was quite acid. A creamy yellow precipitate was obtained which usually flocculated readily. Sedimentation, however, was assisted by the addition of alcohol. The purpose of the excess acid was to redissolve a portion of the protein, which is precipitated in slightly acid solution. After thorough washing with acid-alcohol, it was dried by repeated treatments with absolute alcohol and finally in a desiccator over fused $ZnCl_2$. The preparations obtained in this way were creamy to very light brown in colour, readily ground to a powder, but did not readily dissolve even in alkali. An intense dark blue colour was given with iodine. The ash content varied according to the completeness of the washing with acid-alcohol, but was usually quite low. The nitrogen content varied from 3 to 7 %.

(2) *Attempted purification of crude material.* Many methods of purification were tried and found to be unsuccessful. Precipitation with copper by the addition of Fehling's solution removed only a small portion of the admixed protein. Enzymic digestion both with pepsin and trypsin at the appropriate p_H lowered the nitrogen content somewhat; the best preparation, however, at the end of 10 days' treatment with pepsin still contained 1·9 % N. Fractional precipitation by cautious addition of acid and by the use of protein precipitants under slightly acid conditions proved no more successful owing to the very similar precipitation properties of the protein and carbohydrate.

More successful attempts were made at precipitation under alkaline conditions since the proteins are thereby kept in solution. To a filtered 2 % alkaline extract was added 1 volume of 90 % alcohol, whereupon a white flocculent precipitate appeared. This was filtered off, washed by sedimentation, first with alkaline alcohol to remove protein and then with acid alcohol to remove inorganic salts. It was finally dried with increasing concentrations of alcohol and over fused $ZnCl_2$ in a vacuum. The preparation contained ash, 0·57 %, nitrogen, 0·84 % and yielded sugar equivalent to 92·9 % anhydroglucose on hydrolysis with N H_2SO_4 for 16 hours. It was by far the purest preparation obtained up to this point. To the filtrate from it was added a further 2 volumes of alcohol and the white flocculent precipitate washed as before and dried. It was found to contain even less N, the ash being 1·36 % and N, 0·48 %. These two fractions, however, do not represent all the carbohydrate in the extract. This method of separation proved satisfactory in practice when amended by accomplishing the initial precipitation with at least 2 volumes of alcohol. In this way one fraction only is obtained, since hydrolytic studies had indicated the similarity in all but nitrogen content between the fractions precipitated by 1 and 3 volumes of alcohol respectively. This fraction will be referred to as Fraction B and probably corresponds to the callose of Mangin [1890].

(3) *Properties of Fraction B.* From fresh tissue preparations can be obtained which are colourless and dry readily to a fine powder. Once dried, they only redissolve with difficulty in alkali even on warming. This fraction was insoluble in water and acids and gave an intense blue coloration with iodine. After hydrolysis with acid, glucose was the only sugar detectable. The specific rotation could be determined in alkaline solution. (A solution of concentration 0·44 % in 3 % NaOH had a rotation $[\alpha]_D = +85{\cdot}1°$.) All attempts to remove the small amount of nitrogen still remaining have failed. That it is combined protein is possible, but not probable, in view of the variation in nitrogen content of preparations obtained by similar though not identical means. These have varied from 0·48 to 1·53 %. It was hoped that repeated precipitation from alkaline solution by 1 volume of alcohol might yield a product free from nitrogen, but this process does not seem to be effective and has a definite disadvantage in that the repeated re-solution in alkali, which is necessary, adversely affects the colour and texture of the precipitate. The best preparations are apparently those obtained by an initial precipitation with alcohol, washing by sedimentation first with alkaline alcohol of similar strength, and then with acid alcohol to remove inorganic salts. The possibility of the nitrogen being present not as protein, but as a hexosamine constituent of the carbohydrate, was considered. 5 g. of a preparation containing 1·53 % nitrogen were hydrolysed with concentrated HCl for 3 hours, diluted and the HCl distilled off under reduced pressure. After decolorising with norite the brownish solution was concentrated to a very small volume, cooled and allowed to stand. A small dark sediment appeared. This was filtered off, redissolved in a small quantity of water, and reprecipitated by the addition of dioxan. A very small amount of a white precipitate was obtained insufficient in amount for characterisation, but it was not hexosamine since it exhibited no reducing power in alkaline-copper solution. It would seem therefore that the nitrogen found in Fraction B is due not to hexosamine but solely to protein.

(4) *Hydrolysis of Fraction B.* (*a*) *By acids.* The hydrolysis of this polysaccharide fraction is readily accomplished by boiling with *N* H_2SO_4; less concentrated solutions did not give as complete hydrolysis even on boiling for prolonged periods. While the curves obtained for liberation of reducing sugar from various preparations are similar in general shape, curious differences were noted in the rate. The source and treatment of the preparation seemed inexplicably to affect the rapidity of hydrolysis. Some typical figures are given in Table III. The sample hydrolysing most rapidly and completely was No. 1, which had the lowest nitrogen content. This particular sample was physically in the best condition and on boiling with acid gave an opalescent and "starchy" solution. The other samples on boiling did not go uniformly into solution, even if previously ground and sieved so as to pass a 100-mesh sieve, but gave a more or less brownish suspension from which particles would separate out on standing. It seems reasonable to conclude that the presence of protein modifies the hydrolytic action for physical reasons. Some indication

of the correctness of this assumption was obtained by comparing the rate of hydrolysis of two samples of the same preparations, the one suspended in *N* acid and immediately boiled, while the other was previously boiled for 18 hours with water before bringing up to the same acid concentration. These two samples are respectively 3 i and 3 ii in Table III and it will be seen that the aqueous pretreatment by assisting penetration of the acid favourably influenced the rate of hydrolysis in the opening stages.

Table III. *Acid hydrolysis of alkali-soluble carbohydrate with* N H_2SO_4.

% reducing sugar calc. as glucose.

Preparation No.	Source and tissue	N content %	Time of boiling in hours 0·5	1	1·5	2	3	4	5	6	7	8	9	11	13	16	24
1	Fresh	0·84	61·0	79·5	83·0	85·3	—	—	96·3	102·0	102·5	—	—	—	—	103·3	—
2	Fresh	1·45	35·7	47·7	59·6	—	67·0	70·6	74·1	76·0	—	78·0	—	—	—	—	—
4	Fresh	1·6	34·7	48·5	—	57·2	58·3	—	72·8	—	—	73·9	—	—	79·1	—	—
3 i	Alcohol-extracted	1·53	24·2	39·4	47·6	—	52·0	58·6	—	—	68·7	—	—	73·2	—	—	—
3 ii	Alcohol-extracted	1·53	30·3	41·0	—	46·3	53·9	59·6	—	67·4	—	—	74·5	—	—	—	76·3
5	Fresh	0·95	42·2	56·0	—	71·7	74·6	—	80·8	—	80·8	—	—	—	—	—	81·1

The percentage recovery from these fractions on hydrolysis is far from satisfactory in most cases. This may be due to the presence of some undetected constituent unit, or possibly to incomplete hydrolysis owing to the presence of protein as suggested above. That the latter is the more probable explanation is indicated by the fact that the preparations with the lowest nitrogen contents, namely 1 and 5, gave the most complete recovery.

The only sugar to be identified in the hydrolysis liquid was glucose, characterised by means of its osazone. Tests for mannose, galactose, pentose, and uronic acid were negative. Confirmatory evidence for the presence of glucose was obtained by determining the rotation of the sugar obtained on hydrolysis as described earlier. A solution containing 0·0258 g. sugar per cc. calculated as glucose had a rotation of + 2·59° in a 2 dm. tube, $[\alpha]_D = + 50{\cdot}2°$, a close approximation to the theoretical for glucose.

Some information as to the type of linkage in the molecule was obtained by employing the method of interrupted hydrolysis developed by Norman [1929] in work on gums. The hydrolysis of all these preparations of Fraction B is most rapid in the first hour or so, after which the rate falls off sharply. Accordingly a sample of preparation 2 was taken and boiled for 1 hour with *N* H_2SO_4. Sugar equivalent to 42 % anhydroglucose was liberated. Three volumes of alcohol were added to the hydrolysis liquid at this point and the precipitate of partly hydrolysed material filtered off, washed with acid alcohol, and dried. This again was hydrolysed with *N* H_2SO_4. It was found that the initial rate of liberation of sugar was distinctly less than that of the original sample. Except for a slight lag due to the physical condition, the curve obtained coincided almost exactly with that of the original sample after 1 hour

of hydrolysis. These results are shown in Fig. 1. Curve 1 is the normal hydrolysis curve for the original sample, while curve 3 represents the figures obtained from the hydrolysed sample recalculated on the basis of the 58 % remaining at the end of the preliminary hydrolysis and plotted from the 1 hour point at which hydrolysis was recommenced. The close similarity between this curve and that of the original material is thereby apparent.

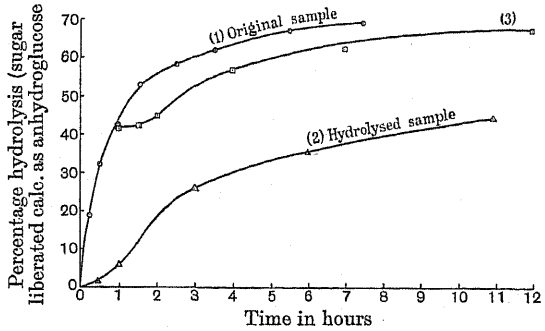


Fig. 1.

The inference therefore is that this polysaccharide contains two types of sugar linkage, one readily hydrolysed by N H_2SO_4, and the other more resistant.

(*b*) *By enzymes.* The hydrolysis of this polysaccharide by enzymes was also investigated. A preparation containing 1·45 % N was ground up to pass a 100-mesh sieve. Four samples of 0·5 g. each were taken and suspended in 50 cc. of water and boiled for a short time. After cooling, 10 cc. of a 2 % enzyme solution were added to each and the mixtures incubated in the presence of toluene. From time to time small aliquots were withdrawn, filtered and the reducing sugar present determined. The following enzyme

Table IV.

	Sugar liberated calc. as glucose (%)	
Enzyme	24 hours	96 hours
Malt diastase	16·9	17·3
Takadiastase	27·6	39·4
"Luizyme"	19·9	22·8

preparations were employed: (*a*) "pangestin," 37°, (*b*) malt diastase, 53°, (*c*) takadiastase, 53°, (*d*) "luizyme,"[1] 37°. The activity of the enzymes was verified under identical conditions on starch. The first named had practically no action on the suspended mould carbohydrate, but the remainder all caused the liberation of some reducing sugar, due correction being made for sugar from the enzyme preparation itself.

[1] A commercial product believed to be obtained from fungal material.

The actual nature of the sugar liberated has not as yet received attention. It is significant that takadiastase and "luizyme," both enzymes prepared from *Aspergillus oryzae*, are active in digesting this mould carbohydrate, but it was somewhat surprising to find that a malt diastase preparation should be active.

(5) *Preparation and properties of Fraction C.* If to the alkaline-alcoholic filtrate from the precipitation of Fraction B acid were slowly added, a cream-coloured flaky precipitate appeared at about p_H 6·2. Further acidification did not cause the separation of any more material, though the solution exhibited a very strong buffer effect at about p_H 4·7. The precipitate obtained at p_H 6·2 had a greyish-brown colour when dried and consisted largely of protein. The nitrogen content was between 10 and 11 %, indicating about 66 % protein. Glucose can be obtained on hydrolysis with N H_2SO_4 indicating the presence of a polysaccharide. Whether this is in combination with protein or not remains to be seen. The fact that the nitrogen content is rather consistent in different preparations might suggest that such is the case. This fraction has not been further investigated.

Summary.

1. An examination has been made of the carbohydrates of *Aspergillus fischeri* grown on a glucose-ammonium nitrate medium. But for a trace of pentose, the only sugar units detectable were glucose.

2. The chief water-soluble carbohydrate was precipitated by acid-alcohol. It gave no colour with iodine, was readily hydrolysed by boiling with 3 % H_2SO_4 and passed slowly through a cellophane membrane. It had a rotation of $[\alpha]_D^{22°} = +86·2°$. Glucose only was obtained on hydrolysis.

3. Crude alkali-soluble preparations containing a high percentage of protein were obtained by extraction with alkali and precipitation with acid. Attempts at purification, by copper precipitation, enzymic digestion and fractional precipitation under acid conditions were unsuccessful.

4. By precipitation with alcohol from alkaline solution a carbohydrate of low nitrogen content was obtained. It gave an intense blue colour with iodine, and had a specific rotation of + 85·1°. The residual nitrogen was present as protein, not as hexosamine. Takadiastase, malt diastase and "luizyme" all liberated reducing sugar.

5. An interrupted hydrolysis indicated the presence of two types of sugar linkage, one easily hydrolysed and the other more resistant. Glucose only was obtained on hydrolysis.

6. The filtrate from the preparation of this alkali-soluble fraction yielded on acidification precipitates containing about 66 % protein and the remainder carbohydrate. These have not been investigated.

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CCXXXI. THE CHEMISTRY OF MOULD TISSUE.
II. THE RESISTANT CELL-WALL MATERIAL[1].

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THE cells walls of fungi lack the structure exhibited by those of higher plants, and at the same time are markedly inert towards the action of most reagents and stains. Many histological investigators, towards the close of the last century, occupied themselves with speculating on the nature of the cell-wall constituents. Tests for the usual structural constituents of plants were indefinite. Most of the workers assumed cellulose to be present, though so altered by combination or infiltration with other materials as to be difficult of characterisation. Several names were given to the cell-wall substance, the most general being "fungus cellulose." A chemical examination of this substance was made by Winterstein [1894; 1895] who showed it to be chitinous in nature. His investigations were mainly on material from higher fungi and his analysis showed about 3·6 to 3·9 % nitrogen in his preparations. From *Boletus edulis* Schöll [1908] obtained, by exhaustive extractions with hot 10 % KOH solution, a material which he described as pure chitin, yielding on hydrolysis 78 % of crystalline hexosamine hydrochloride. He concluded that the cell-walls of this organism are either composed of pure chitin or of chitin in very loose combination with a nitrogen-free carbohydrate. Later Sumi [1929] obtained chitin from the walls of spores of *Aspergillus oryzae*. He considered it to be present there in association with other substances. Proskuriakow [1926] has prepared from *Boletus*, *Agaricus* and *Polyporus*, by repeated extraction with 10 % NaOH followed by treatment with a solution of potassium permanganate, preparations of high nitrogen content (6·1–6·3 %). The actual amount of such material in the fungi is apparently small, since the yield from *Agaricus* was 5 % and from *Polyporus* 3–5 %. Takata [1929] by similar means obtained from *Aspergillus oryzae* 3·18 % of "chitin" containing 6·37 % N.

The purpose of these investigations was to examine the cell-wall material from *Aspergillus fischeri*, in comparison with that from higher fungi.

[1] This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

Experimental.

Preparation.

After prolonged alkaline extraction of the tissue, a cream-coloured residue remained. This after careful washing and dehydration with alcohol and ether dried to a colourless mass and was readily powdered. If dried without alcoholic dehydration, or if this treatment was not exhaustive, the residue on drying became grey or brownish in colour, hard and vitreous in character and almost impossible to grind to a powder. The nitrogen content of this material varied according to the violence of the alkaline treatment employed, but was in general rather under 3·5 %. Some figures obtained for nitrogen content and the treatment employed are given below. These are not all however from the same batch of mould mycelium.

	% nitrogen	% ash
1. Boiled with 60 % NaOH for 1 hour	2·69	0·26
2. Boiled with 25 % KOH for 2 hours	2·78	—
3. Boiled twice with 10 % NaOH for 48 hours each time ...	3·10	0·59
4. Boiled with 5 % NaOH for 3 hours after pre-treatment with more dilute alkali	2·41	1·22
5. Boiled with 4 % NaOH for 1 hour	2·35	—
6. Boiled with 2 % NaOH for 3 hours	2·53	0·37

Tissue, which had been previously treated with a fat solvent, saponified with alcoholic NaOH and then exhaustively extracted with hot NaOH solution yielded about 20 % of this resistant fraction. When such a preparation was boiled for 2 or 3 hours with concentrated HCl, diluted and concentrated under reduced pressure to remove HCl, decolorised with norite and again concentrated to a small volume, crystals separated out which were identified as hexosamine hydrochloride. (N found, 6·45 %; theoretical, 6·49 %.) With phenylhydrazine the crystals gave glucosazone, and they titrated nearly quantitatively as glucose with the Shaffer-Hartmann alkaline copper reagent. (1·005 mg. hexosamine hydrochloride titrated as 0·805 mg. glucose; theoretical, 0·835 mg. glucose.)

The presence of hexosamine in the resistant fraction of the cell-wall of fungal mycelium has long been recognised and was, of course, responsible for its description as "chitin." Its identity with chitin from animal sources, however, must not be assumed. The nitrogen content of all these preparations was about 3 %; even after the most violent alkaline treatment, namely by boiling for 4 days with 10 % NaOH, it was not increased above 3·1 %. Chitin from animal sources contains over 6 %. The fungal preparations of Proskuriakow [1926] similarly contained over 6 % N. Since anhydrohexosamine ($C_6H_9O_4.NH_2$) has a nitrogen content of 8·7 % it is evident that the resistant complex in the case of *Aspergillus fischeri* is only in part composed of hexosamine units. A nitrogen content of 3·0 % would indicate only 34·5 % anhydrohexosamine. The nature of the remainder of this complex has not been determined. Some workers have referred to the presence of cellulose in

the cell walls of fungi, and Thomas [1928] claims to have demonstrated its presence in *Fusarium*. His data, however, seem hardly sufficient, since materials other than cellulose are soluble in ammoniacal copper solution. The evidence to be presented below indicates that cellulose, as ordinarily found in plants, is not present. Combination with hexosamine units, of course, might possibly so alter its character and properties as to make it unrecognisable, but even this is unlikely in view of certain results obtained on hydrolysis with dilute acids.

Properties.

This resistant residue was wholly insoluble in Schweitzer's cuprammonium solution and in a saturated solution of zinc chloride in HCl, both solvents for cellulose. When ground to pass a 100-mesh sieve it was, however, readily and completely soluble in 72 % H_2SO_4 in the cold giving a slightly brown liquid. A xanthate was obtained by stirring in CS_2 after treatment with 17 % NaOH solution in the cold. The xanthate had a typical golden-brown colour and gave a smooth thick paste. On dilution and acidification the original material separated out. It had, however, no tensile strength either as a film or as a fibre. This residue was carefully washed with water and dried with repeated changes of alcohol. The nitrogen content was found to be unchanged by the process.

Acetylation.

In order to demonstrate the identity of celluose, should it be present in this fraction, several attempts were made at acetylation. 10 g. of tissue (N, 2·56 %) ground to pass a 100-mesh sieve were treated with 37·5 cc. of glacial acetic acid, 37·5 cc. of acetic anhydride and 1 cc. conc. H_2SO_4 as catalyst. After stirring for 2 minutes, the mass was allowed to stand for 30 minutes in a cold water-bath. The solution was then again stirred for 4·5 hours, diluted with 100 cc. glacial acetic acid and filtered. Very little appeared to have gone into solution. The clear filtrate was poured into 500 cc. distilled water and allowed to stand for several hours. A slightly milky emulsion was obtained on the addition of chloroform. The chloroform layer on separation was concentrated in the presence of a little water, upon which a granular white precipitate appeared in the water. This was centrifuged, washed several times in water and dried in a vacuum oven at 60°. The yield was very small, amounting to only 0·2 g. The acetyl content was determined by the method of Murray, Staud and Gray [1931] by solution in pyridine and treatment with NaOH at 53° for $\frac{1}{2}$ hour. (CH_3CO obtained 36·1 $\pm$ 1·5 %; theoretical for diacetylhexose anhydride, 34·9.) The residue was thoroughly washed and then suspended in water and extracted in a continuous extractor with *iso*propyl ether in order to remove all traces of free acetic acid. After drying with alcohol in a vacuum oven at 60°, the acetyl content was determined and found to be 5·8 %.

Acetylations were repeated under different conditions in an attempt to

obtain a product of higher acetyl content. By treatment of 10 g. of the resistant material with 0·5 g. $ZnCl_2$, 40 cc. acetic anhydride and 40 cc. glacial acetic acid at 50° for 24 hours with continuous stirring, an insoluble product containing 6·02 % acetyl was obtained, but no soluble product as in the first experiment.

Barnett's [1921] method as modified by Irvine and Hirst [1922] with another sample gave an insoluble product containing 10·7 % acetyl, but only a trace of soluble product. This method involves the use of $SOCl_2$ as a catalyst, the temperature being maintained at 65° for 18 hours, and is the most effective method for acetylation of cellulose, which, under these conditions, goes readily into solution and gives the triacetate. Since no soluble product was obtained, the conclusion seems justified that cellulose in its normal condition was absent. No attempt was made to determine the position of the acetyl groups in the acetylated material. Even on the hypothetical basis of the formation of a mono-acetyl anhydrohexose or hexosamine, calculation shows that there has only been the introduction of one acetyl group for every two hexose or substituted hexose units in the molecule. This resistant structural material is clearly not easily acetylated.

Attempts were made to isolate substituted fission products by acetolysis. 10 g. tissue were treated with 40 cc. acetic anhydride and 5 cc. conc. H_2SO_4 in the cold, and allowed to stand at room temperature for prolonged periods. It was not found possible to isolate any product. In the cold the material failed to go into solution, and if warmed, charring and decomposition occurred.

Hydrolysis.

As ordinarily obtained, the ash of this material was very low, while the nitrogen present indicated from 33–40 % anhydrohexosamine. Hydrolysis can be effected with concentrated H_2SO_4, but the recovery of reducing sugar expressed as glucose is far from complete. The hydrolyses were carried out as follows. About 0·5 g. of material passing a 100-mesh sieve was stirred with 10 cc. 80 % H_2SO_4 till completely dissolved, the beaker being kept cold during this process. After standing for varying periods in an ice-box, it was diluted to 200 cc. and the solution then autoclaved at 15 lb. for 1 hour. Reducing sugar was determined by the Shaffer-Hartmann method. Although the hydrolysis conditions were varied extensively, the highest yield of apparent sugar obtainable was 72 % calculated as anhydroglucose. The results obtained by this means were erratic, whereas good recovery can readily be obtained from cellulose. A small quantity of residue remained undissolved at the close of the hydrolysis. This was very difficult to determine accurately since washing was difficult and charring took place on drying. The amount varied from 5–10 %.

Acid hydrolysis under much milder conditions liberated appreciable quantities of reducing sugar. Table I indicates the percentage of apparent glucose from this resistant material on boiling with N and $N/2$ H_2SO_4.

Table I. *Hydrolysis of resistant cell-wall material.*

Time in hours	Percentage apparent glucose liberated		Time in hours	Percentage apparent glucose liberated	
	N H_2SO_4	$N/2$ H_2SO_4		N H_2SO_4	$N/2$ H_2SO_4
½	8·6	2·8	15	33·5	—
1	8·9	4·7	23	38·8	—
3	15·9	10·9	28	—	30·1
4	20·3	14·0	32	—	30·7
6	25·7	15·6	39	51·1	—
8	—	19·6	48	—	37·2

The nitrogen liberated during the course of this hydrolysis was estimated only at the end of 24 hours and 39 hours with N H_2SO_4.

Table II. *N liberated on hydrolysis with* N H_2SO_4.

Time in hours	Total N %	N liberated from 100 g. g.	Calculated as hexosamine
24	2·77	1·44	18·4
39	2·77	1·75	22·4

The important fact which emerges from these results is that both the nitrogenous (chitinous) part and the non-nitrogenous part of this complex are hydrolysed slowly by dilute acid treatment. In order to investigate more fully the relative rates of hydrolysis, a further series of experiments was carried out under pressure and with a number of acid concentrations. By this means the charring which took place on long boiling with acid was avoided, except in two or three cases when the autoclave treatment was prolonged. A weighed amount of material (0·5 g.) was placed in a tube with 30 cc. of acid of the required strength, autoclaved at 14 lbs., and then made up to 50 cc. After being allowed to settle, aliquot portions were taken for the determination of apparent glucose, and soluble nitrogen, subsequently calculated as hexosamine. The residue was then filtered through a Gooch crucible, thoroughly washed, dried and weighed. The original resistant material contained N 3·1 % and ash 0·59 %. The results are presented in Fig. 1.

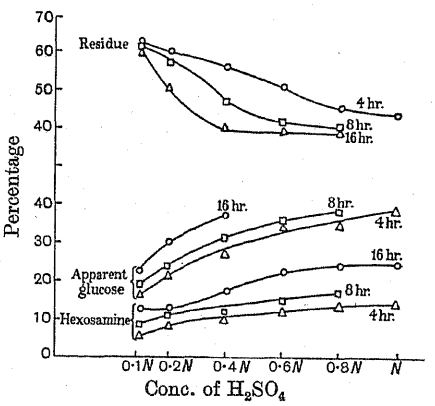


Fig. 1. Hydrolysis of resistant material with various strengths of H_2SO_4 at atmospheric pressure.

The evidence here presented indicates the very resistant nature of at least a portion of this residue; N H_2SO_4 at one atmosphere pressure removed 55 % in 4 hours and only an additional 8 % if the treatment was prolonged for a further 12 hours. Even then only 71·3 % of the nitrogen had been liberated. The original material on a basis of 3·1 % N contained 35·6 % anhydrohexosamine, while the residue at the end of 16 hours' treatment with N H_2SO_4

contained a very similar percentage, namely 34·7 % (1·12 g. N in 37·1 g. material). It should not, however, be deduced from this that the hydrolysis of the nitrogenous and non-nitrogenous units of this complex proceeded in parallel. This was definitely not the case for, in the early stages and under milder conditions, glucose or non-nitrogenous material was removed more rapidly than hexosamine. The approximate composition of the residue obtained throughout the 4-hour and 8-hour hydrolyses may similarly be calculated from the nitrogen not in solution. This is given in Table III. From this

Table III. *Approximate composition of hydrolysis residue.*

	4 hours				8 hours			
Concentration of acid	Residue %	Nitrogen in residue	Calculated as anhydrohexosamine	Anhydrohexosamine in residue %	Residue %	Nitrogen in residue	Calculated as anhydrohexosamine	Anhydrohexosamine in residue %
	45·1	1·98	22·7	50·3	—	—	—	—
0·8 *N*	46·2	2·0	23·0	49·9	41·5	1·72	19·8	47·7
0·6 *N*	52·0	2·13	24·5	47·2	43·0	1·86	21·4	49·8
0·4 *N*	57·1	2·30	26·4	46·2	47·8	2·13	24·5	51·3
0·2 *N*	60·7	2·44	27·1	44·7	58·5	2·20	25·3	43·3
0·1 *N*	63·5	2·67	30·7	48·4	62·3	2·44	28·0	45·0

it will be seen that practically one-half of the residue obtained in these two series was composed of anhydrohexosamine units. The remainder, however, cannot be considered as consisting solely of anhydroglucose units, though glucose was the only sugar detected as an osazone. The recovery throughout these hydrolysis experiments was far from complete and averaged about 80 %. By analogy with chitin from animal sources the presence of acidic groups was suspected, and this possibility was therefore examined. 1·362 g. of finely ground material were dissolved in 20 cc. 80 % H_2SO_4, allowed to stand for 3 hours in an ice-box and diluted to 400 cc. The solution was autoclaved for 1 hour and distilled in steam, 4 litres of distillate being collected. The distillate titrated equivalent to 29·4 cc. 0·103 *N* NaOH. The volatile acid was characterised as acetic by the Virtanen-Duclaux determination. The residue from the distillation of volatile acid was extracted in a continuous extractor with *iso*propyl ether for non-volatile acid. The extract when taken up with water contained only a trace of acid. It would seem, therefore, that acetic acid is liberated on hydrolysis in appreciable amount and must be considered as a constituent of the molecule. The sample of resistant material used in the above experiment contained 0·37 % ash and 3·55 % N; CH_3CO content, 9·55 %. The anhydrohexosamine content ($C_6H_9O_4.NH_2$) calculated from the nitrogen present was 40·8 %. Since, in the case of chitin from animal sources, the acetyl groups have been shown to be linked to the nitrogen of the glucosamine, it is not unlikely that a similar relationship may hold in this case. The acetyl content found corresponds very nearly to that required if there is one CH_3CO group for each hexosamine unit.

From this evidence obtained by hydrolysis it is not possible to make any very definite statement as to the nature of the resistant material. It is very improbable that a single entity is present. The changing ratio during hydrolysis between the hexosamine and the non-nitrogenous portion of the complex suggests the presence of two components hydrolysing at different rates. The fate of the acetyl groups in mild acid hydrolysis has not been determined, but the apparent production of a material containing about 50 % anhydrohexosamine with widely different concentrations of acid is significant. These hydrolytic studies provide further evidence for the absence of cellulose, which, since it would be unaffected by the low concentrations of acid employed, would cause the ratio of the nitrogenous to the non-nitrogenous part of the complex to fall rather than to rise, as was observed. Tentatively it is suggested that this resistant cell-wall complex has two components. The major one consists of one molecule each of hexosamine, glucose and acetic acid, the last probably being linked to the nitrogen as in animal chitin. The other consists only of glucose units, is more readily hydrolysed and is soluble to some extent in strong alkali. Their association may be compared to that between xylan and cellulose in many plant fibres.

Fermentation of the resistant residue.

Still further to investigate the possible presence of cellulose in this fraction, portions of it were fermented with cellulose-decomposing organisms of high activity. 200 g. of wet residue (26·6 g. dry matter) were suspended in 500 cc. of a mineral salt solution and 5 g. of $CaCO_3$ added. After prolonged sterilisation, a small quantity of sterile peptone was added (0·5 %) and the whole shaken and inoculated with a heavy inoculum of the desired organism. Two fermentations were carried out (A) with a "purified" culture of thermophilic cellulose-decomposing organisms—very active in cellulose fermentation but containing several forms, mainly short rods, (B) with a purer culture containing the cellulose organism isolated by Snieszko (unpublished work) together with its two concomitant contaminants, which together bring about a rapid and extensive decomposition of pure cellulose.

Both fermentation mixtures were incubated at 55° for a period of 3 weeks. The fermentation was obviously very slight. At the conclusion of this period the volume was adjusted and aliquots taken for determination of the unfermented residue. These were washed with dilute HCl to remove carbonate and then thoroughly with water. The fermentations were also examined microscopically to verify the cultures employed. The loss of organic matter was found to be slight; in A, 10·72 % and in B, 8·76 %. While this cannot be taken as evidence for the absence of cellulose, it at least indicates that it is present only to a small extent, if at all, for under similar conditions pure cellulose would have been almost completely decomposed by these cultures in a much shorter period of time.

SUMMARY.

1. After exhaustive alkaline treatment of the tissue of *Aspergillus fischeri*, about 20 % of a resistant cell-wall material remained.

2. The preparations yielded hexosamine hydrochloride on hydrolysis with HCl and had a nitrogen content of about 3 %. This indicated the presence of approximately 35 % anhydrohexosamine in the complex.

3. The material was insoluble in cellulose solvents, but gave a xanthate.

4. Only a trace of a soluble acetylated derivative was obtained; the insoluble product of acetylation contained 10·7 % acetyl. Acetolysis resulted in charring and decomposition.

5. Hydrolysis could be effected readily after solution in concentrated H_2SO_4, but the maximum recovery of apparent glucose was only 72 %. Dilute acid liberated both glucose and hexosamine slowly, and under pressure more rapidly. Glucose units were liberated more rapidly than hexosamine in the opening stages, and a residue containing about 50 % anhydrohexosamine was consistently obtained unless the treatment was violent.

6. Acetic acid was obtained on acid hydrolysis in amount approximately equivalent to one CH_3CO group for every hexosamine unit.

7. Fermentation with active cultures of thermophilic cellulose-decomposing organisms was slight, 10 % only being lost in 3 weeks.

8. The evidence suggests that cellulose, in the form usually met with in plants, is absent from this complex, which is probably a mixture of two components, one containing hexosamine, glucose and acetyl units, and the other, more readily hydrolysed, glucose units alone.

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CCXXXII. GLUCOSONE.

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It has been suggested by Hynd [1927] that glucosone is an essential intermediary in carbohydrate metabolism and is partly responsible for the symptoms of insulin hypoglycaemia. The evidence on which these assumptions are based is not very satisfactory, and attempts have therefore been made to detect glucosone *in vivo* and *in vitro* under physiological conditions.

Experimental.

Preparation of glucosone. The original method of Fischer [1889] is lengthy, but a review of other methods by Hynd [1927] provides no good alternative. Evans, Nicoll, Strouse and Waring [1928] describe the production of glucosone by the oxidation of fructose with copper acetate, and a further method is described below.

Riley, Morley and Friend [1932] used selenium dioxide as an oxidising agent in the preparation of substituted glyoxals; they showed that fructose (but not glucose) reduced hot selenious acid, but they did not investigate the products formed.

A solution of 50 g. fructose and 15 g. selenious acid in 200 cc. water was heated for 3 hours under reflux on a boiling water-bath, and on cooling filtered from the reddish deposit of selenium. To the filtrate 70 g. lead acetate in 150 cc. water were added, and the solution after cooling in ice was filtered from the precipitated lead selenite. Ice-cold $Ba(OH)_2$ was now added until the solution was strongly alkaline, and the precipitated lead glucosone compound filtered off and washed thoroughly until free from fructose. From this stage the preparation now followed exactly that of Fischer [1889]. From the syrup so obtained (after purification by dissolution in alcohol and evaporation *in vacuo*) the osazone of glucose was prepared by warming to 37° with phenylhydrazine acetate for 30 minutes, filtering off and recrystallising from alcohol (see Table I).

Table I. *Melting-points.*

(*a*) Glucose phenylosazone from glucose	192–3° uncorr.
(*b*) Glucose phenylosazone from glucosone made by this method	193–4° ,,
(*c*) Mixture of (*a*) and (*b*)	192–3° ,,

Reactions of glucosone. Glucosone gives Schiff's reaction immediately in the cold, and is also bound by sodium sulphite; it therefore probably contains a free aldehyde group. A strong solution gives an immediate precipitate in the cold with 2:4-dinitrophenylhydrazine; the osazone (of glucose) formed crystallises in needles M.P. 252–3° (corr.); Glaser and Zuckermann [1927] give 256–7° "bei raschem Erhitzen." For the detection and estimation of glucosone in dilute solution, an equal volume of the reagent (0·5 g. 2:4-dinitrophenylhydrazine in 30 cc. 2*N* HCl [Case and Cook, 1931]) is added, and the solution allowed to stand at 37° for an hour; further standing is liable to bring down glucose and fructose if these are present. This reaction is delicate, a precipitate being given under these conditions with 0·03 % glucosone solution. Trichloroacetic acid does not interfere.

Glucosone is not appreciably destroyed by evaporation *in vacuo* provided the solution is acid. A small quantity of glucosone was dissolved in 100 cc. blood-serum, and the proteins precipitated with 5 g. trichloroacetic acid. The filtrate was made up to 100 cc., and 20 cc. were withdrawn (solution A). The remainder was evaporated *in vacuo* at 45° to a syrup, and again evaporated after the addition of 50 cc. water; the residue was diluted to 80 cc. and 20 cc. withdrawn (solution B). To solutions A and B 10 cc. of the 2:4-dinitrophenylhydrazine reagent were added, and after standing at 37° for an hour the precipitates were weighed:

Weight of osazone from A	...	...	31 mg.
Weight of osazone from B	...	...	29 mg.

The loss is not greater than the experimental error involved.

Attempted detection of glucosone. In the first series of experiments attempts were made to detect glucosone in the blood of rabbits in hypoglycaemic convulsions following a large overdose of insulin. Details are given in Table II. The insulin was injected intravenously unless otherwise stated.

Table II.

Rabbit No.	Weight kg.		Insulin units	Time of onset of convulsions hours*	Blood obtained cc.
1	2·1		26		
		1 hour later	26	2	20
2	2·8		20†		
		1 hour later	20	4	25
3	2·7		6		
		1 hour later	6	3	15
4	2·2		6		
		1½ hours later	6	1½	10
5	3·3		Control		30

* As from the second injection. † Subcutaneous.

When convulsions occurred the animals were killed by a blow on the head, and rapidly bled from the neck into 10 cc. 100 % (w/v) trichloroacetic acid. The filtrate and washings from the precipitated proteins were evaporated

in vacuo at 45° to small bulk, and an equal volume of 2:4-dinitrophenylhydrazine reagent was added. In no case was any precipitate formed after an hour at 37°. In all cases a deep red coloration, more marked in the insulinised animals than in the control, was observed on adding the reagent; this may be due to the presence of glyoxals other than glucosone. Rabbits 1 and 2 were fed on carrots for 24 hours previous to injection; 3 and 4 were starved for 48 hours previously, while the control was fed normally.

Since these experiments gave a negative result, a further attempt was made to detect the formation of glucosone from insulin and fructose in the presence of liver tissue. The technique employed was essentially that of Neuberg and Gottschalk [1924] and Gottschalk [1925] in a similar investigation. The following flasks were set up:

	A	B	C	D
Liver, g.	100	100	100	100
Calcium sulphite, g.	4	4	4	4
Fructose, g.	2	2	2	2
Insulin, units	40	—	40	—
Antiglyoxalase, mg. (Parke, Davis pancreatin)	—	—	20	20

To each flask were added 100 cc. phosphate buffer at p_H 7·0 and 45 mg. 8-hydroxyquinoline sulphate (neutralised with NaOH) as antiseptic. The flasks were incubated at 37° for 24 hours, a slow stream of air being passed through to stir the contents and render the system aerobic. After deproteinising with trichloroacetic acid, the filtrate and washings were evaporated to small bulk *in vacuo*, 50 cc. water added and again evaporated; any methylglyoxal or acetaldehyde present was thus removed. The solutions were filtered, diluted to 20 cc. and after adding 10 cc. of the reagent were allowed to stand at 37° for an hour. No precipitation occurred.

Discussion.

From the evidence available it does not seem that glucosone plays any important part in intermediary metabolism. The evidence of Hynd [1927] rests on a superficial similarity between the convulsions of insulin hypoglycaemia and those produced by glucosone: it was also found that adrenaline and pituitrin exerted an antagonising influence in both cases. The injection of other glyoxals, however, also causes convulsions: thus Kermack, Lambie and Slater [1927] found that methylglyoxal, and again [1929] that hydroxypyruvic aldehyde were toxic, though the last observation has been disputed by Hynd [1931]. Moreover, a very large dose of glucosone is required to produce the effect; Herring and Hynd [1928] found that 1·7 mg./g. body weight was the convulsant dose for rabbits: calculation shows that the glucosone must be present in the blood (assuming that it is not immediately removed) to the extent of more than 1 %. Unless, therefore, glucosone disappears from the blood very rapidly, the failure to detect it in appreciable quantities (more than 0·01 %) renders it unlikely that the convulsions of insulin hypoglycaemia

are even partly due to its presence. Evidence that glucosone is not rapidly removed from the blood was found by Hynd [1927]; 15 minutes after the injection of a small quantity of glucosone into a mouse, the apparent blood-sugar (determined by MacLean's method, which is affected by glucosone) had risen from 0·161 % to 0·238 %. Kermack, Lambie and Slater [1929] have pointed out that the absence of convulsions when glucose is injected along with insulin renders it improbable that glucosone causes these convulsions. Further, it is curious that the injection of glucose should alleviate the convulsive condition, since in a system glucose $\xrightarrow{\text{insulin}}$ glucosone the increase in the concentration of glucose should increase the production of glucosone correspondingly.

The attempted isolation of glucosone from incubated liver tissue also failed. Antiglyoxalase was added to flasks C and D to prevent the oxidation of glucosone, if it were formed, to gluconic acid, since experiments indicate that glyoxalase promotes this change. (Further work is in progress on this question, since it is contrary to the finding of Levene and Meyer [1915].) There is thus no evidence that glucosone is formed by the liver, even with added insulin; Clift and Cook [1932, private communication] have shown that glucosone does not occur in fresh liver, and we have confirmed this result. It is useless to attempt to account for the discrepancy which has been pointed out [Holden, 1926 and others] between the reducing power and rotation of blood filtrates by the presence of glucosone, since Groen and Meyer [1932] have shown that reduced glutathione is responsible, even in the case of diabetic patients under insulin treatment. The possibility of glucosone acting as an intermediary cannot be excluded entirely, since Walker [1932] has observed that it is formed from glucose by a mould of the *flavusoryzae* group of *Aspergilli*; it has not been found possible, however, to detect it in the animal body.

Summary.

1. A method is outlined for the preparation of glucosone from fructose by oxidation with selenious acid; a method for its detection is also described.

2. Glucosone does not occur to the extent of more than 0·01 % in the blood of rabbits suffering from convulsions caused by insulin, and it seems that this condition is therefore due to the lowered blood-sugar alone, and not to the accumulation of glucosone.

3. No evidence was obtained that glucosone is formed by the liver, even in the presence of insulin, nor does it occur in fresh liver.

4. There is thus no evidence that glucosone is formed in the intermediary metabolism of the animal body.

We wish to express our thanks to Sir F. G. Hopkins, to Dr Hamilton McCombie and to Dr T. S. Hele for their advice and criticism; and to Mr J. O. Giršavičius and Mr F. P. Clift for a number of suggestions.

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CCXXXIII. THE AUTOXIDATION OF DIALURIC ACID.

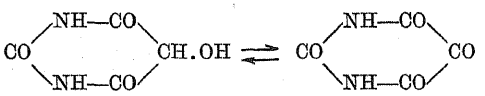
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Of recent years, biological studies of oxidation-reduction have been chiefly concerned with the rate and specificity of the oxidative reactions between metabolites. At the same time, many oxidation-reduction systems have been precisely defined electrometrically by measurements at an inert electrode. It will therefore be interesting to co-ordinate, where possible, the kinetics of oxidation with the oxidation-reduction potentials of the reacting systems.

With this end in view, a previous description of the electrode potentials of the dialuric acid-alloxan system:



included some preliminary data describing the atmospheric oxidation of dialuric acid [Richardson and Cannan, 1929]. The extent to which the reactants would associate to the dimeride alloxantin and interfere in the system was also formulated, but was found to be scarcely significant in dilute solution. The rapid, roughly linear velocity of oxidation obtained was therefore not an unlikely one. At the same time the manifestation in the data of some correlation in acid solution between electrode potential and rate of oxidation made an extended investigation using improved technique highly desirable.

While such experiments were being initiated with a Warburg gas absorption apparatus, the first of a series of three papers dealing with this autoxidation [Hill, 1930; 1931; 1932] appeared. His observations, however, would not submit to an orderly interpretation, and agreed neither with our published findings nor with such gasometric data as were then available. His calculations regarded alloxantin as the end-product of the oxidation, an assumption irreconcilable with the actual direct observations of Thunberg [1915]. Consequently, a much more thorough investigation of this autoxidation than was at first anticipated is now to be reported.

Experimental.

The rate of diffusion of oxygen into a reaction mixture effectively limits the technique available for measuring atmospheric oxidation velocities. In previous experiments we added solutions already saturated with oxygen to dialuric acid at high dilution to overcome this limitation, but the increased liability to error at such dilutions made a different technique desirable. It was also clear that oxidation in other than acid solution was too rapid to allow the accurate withdrawal of samples for titrimetric estimation of velocity. Yet current indicator methods were felt to be poorly suited to defining a continuous series of points during the oxidation. Consequently the gasometric measurement of oxidation in a set of six Warburg absorption manometers was adopted, small quantities of less dilute solutions being exposed to as large a surface of air as possible. Although the apparatus is not well suited to following oxidations with the rapidity that was attempted, the application of extensive controls should have minimised fortuitous errors. An abundance of fairly reproducible observations has been accepted as the practical criterion of reliability.

The preparations of dialuric acid, H_2O; alloxan, H_2O; and alloxantin, $2H_2O$ used were identical with those employed for the previous investigation. Stock solutions of dialuric acid and alloxantin in 0·05 *N* HCl were made up in sufficient quantity to last 2 or 3 days and were stored under hydrogen for immediate delivery from a burette into the absorption flasks. Solutions were approximately saturated at room temperature (0·025 *M* dialuric acid, and 0·0083 *M* alloxantin), and 2 or 3 times daily were titrated directly against 0·01 *M* iodine solution with starch indicator [*cf.* Biilmann and Bentzon, 1918]. Titratable reducing power never changed by more than 3·5 % on storing, and was seldom smaller than 95 % of the value calculated by weight. In the calculation of results, the iodine titre is accepted as the measure of dialuric acid concentration.

Stock buffer solutions adopted were 0·1 *M* and 0·05 *M* HCl, 0·1 *M* citrate, and Prideaux and Ward's universal buffer mixture 0·1 *N* with respect to each constituent group (this is of greater strength than is customary, care being necessary in cold weather lest the phenylacetic acid should become supersaturated in acid stocks). Buffer p_H values were measured with a Hildebrand hydrogen electrode, a saturated KCl calomel half-cell and a Cambridge portable potentiometer.

Experiments were conducted in the Warburg apparatus under the following conditions.

Temperature of thermostat, 18°; manometer liquid, Brodie's fluid (density $1{\cdot}032_2$); average capillary diameter 0·35 mm.; amplitude of swing, about 6 cm.; number of oscillations per min., about 120; capacity of flasks to manometer level, (1) 20·1 cc., (2) 19·5 cc., (5) 23·2 cc., (6) 20·3 cc. (by weight of mercury); volumes of experimental solutions, 2·4 and 1·2 cc.; average exposed

area of base of flasks 8·7 cm.^{2}; average depth of liquid, 2·8 and 1·4 mm. All flasks in use were of the same general dimensions, and were constructed in the usual conical shape with side-arm and NaOH tube.

For each experiment, the stock solutions were delivered directly into the flasks from micro-glassware. The proportions were decided so that the reacting mixtures should contain: dialuric acid of the required concentration, the exact equivalent of 0·1 *M* NaOH to bring the dialuric acid solution to the desired p_H, as large an excess of buffer solution as possible, comparable concentrations of total salts, added catalytic solutions where desired, and, finally, distilled water to the required volume. Table I describes the solutions used between p_H 4 and 11. At more acid p_H values, the proportions were suitably modified

Table I. *Constitution of reaction mixtures.*

Stock reductant: 0·025 *M* dialuric acid (or 0·0083 *M* alloxantin) in 0·05 *M* HCl.

	Conc. of dialuric acid					Conc. of alloxantin	
	0·0166 *M*	0·0125 *M*	0·0066 *M*	0·0063 *M*	0·0021 *M*	0·0055 *M*	0·0021 *M*
Reductant (cc.)	1·6	1·2	0·4*	0·6	0·2	1·6	0·6
0·05 *M* HCl (cc.)	—	—	—	0·6	1·0	—	0·6
0·1 *N* buffer (cc.)	0·6	0·75	0·5	0·75	0·75	0·6	0·75
0·2 *M* NaOH (cc.)	—	0·45	0·14	0·375	0·325	—	0·35
0·8 *M* NaOH (cc.)	0·15	—	—	—	—	0·133	—
Water + catalyst (cc.)	0·05	0	0·16	0·075	0·125	0·067	0·1
Total volume (cc.)	2·4	2·4	1·2	2·4	2·4	2·4	2·4
Buffer strength	0·025 *N*	0·031 *N*	0·042 *N*	0·031 *N*	0·031 *N*	0·025 *N*	0·031 *N*
Ionic strength (p_H 8)	0·15	0·16	0·19	0·16	0·15	0·14	0·15

* 0·02 *M* dialuric acid stock.

for concentration of HCl un-neutralised and of dialuric acid un-ionised at the desired p_H (dialuric acid $p_K = 2·8$). Where catalyses were attempted, the quoted catalytic strengths were obtained by dilution from stock solutions of 0·02 *M* ferrous sulphate in 0·005 *M* HCl, 0·1 *M* KCN (from 95 % KCN solid), and 0·01 *M* barbituric acid (all by weight). The acidity or alkalinity of the added solutions was included in estimating the NaOH additions required above.

In initiating an experiment the main body of the flask received the dialuric acid, HCl and distilled water, the other constituents being added to the side compartment. Thus dialuric acid solutions were stored at optimum stability awaiting immediate admixture after temperature equilibration. Where it was necessary, the NaOH tube received 0·05 cc. 40 % NaOH on filter-paper (of vol. displacement 0·025 cc.) as CO_2 absorbent. After temperature equilibration, the manometers were reset to the standard levels, the gas system closed, and the solutions mixed by tilting at zero time. The method, allowing the rapid admixture of fully aerated solutions not confined in narrow tubes, permits estimation of zero time to within a few seconds. It also obviates the presence of metal parts of doubtful catalytic inertness. At the same time the degree of vagueness as to final p_H which it certainly permits is overcome by the large number of experiments conducted.

The course of the reaction was followed by manometer readings at intervals appropriate to the rapidity of absorption, barometric fluctuations being ascertained from a control manometer. As the half-life period of dialuric acid varied from more than 75 min. down to 1 min., the faster reactions were necessarily followed only singly or in pairs. Nevertheless, certain observations obviously refer to rates outside the limits of effective measurement of the apparatus and can be traced as such in the data.

The maximum oxygen requirement for the formation of alloxan and H_2O_2 from the solutions is theoretically 0·9 cc. This is 24 % of the total oxygen of the flasks. Actually, however, unregulated initial oxidation will have somewhat diminished this requirement. Nevertheless, since the acid solutions were exposed to air before buffer admixture only for some 7 to 10 mins., during which time oxidation with even vigorous agitation will be found not to exceed 20 to 30 %, preliminary oxidation with the apparatus stationary cannot have been large.

Calculation of results.

Notation.

T = absolute temperature in ° C.

α_{18} = solubility of the reactive gas in cc. per cc. solution.

V_s, V_a, V_g = volumes in cc. of the solution, the CO_2 absorbent, and the gas space (empty), respectively.

C = a constant for each flask and each value of $(V_s + V_a)$.

h = alteration of pressure in mm. Brodie's fluid.

$X = hC$ = oxygen uptake in cc. (at 0° and 760 mm.).

n = g.-mol. oxygen absorbed per g.-mol. dialuric acid.

m = intended molarity of dialuric acid.

y = correction factor determined by iodine titration.

x = degree of oxidation $= \dfrac{X}{\text{theoretical } O_2 \text{ uptake}} = \dfrac{X}{22{\cdot}4 V_s nmy}$.

t = time in minutes.

$k = \dfrac{x}{t} = \dfrac{X}{22{\cdot}4 V_s nmyt}$ = linear velocity constant of oxidation.

Procedure.

In the Warburg (constant volume) method using Brodie's fluid in the manometer, the gas absorption is related to the pressure change by the following formula:

$$X = \frac{h}{10{,}000}\left[(V_g - V_s - V_a)\frac{273}{T} + V_s\alpha_{18}\right] = hC.$$

For flask No. 1, using 2·4 cc. solution and the adopted CO_2 absorbent, this reduces to:

$$X = \frac{h}{10{,}000}\left[(20{\cdot}1 - 2{\cdot}4 - 0{\cdot}075)\frac{273}{291} + 2{\cdot}4\,(0{\cdot}031^*)\right] = 1{\cdot}660h \times 10^{-3}.$$

* Since the partial pressures of nitrogen and water vapour are constant during the reaction, this term should comprise just the solubility of oxygen in the aqueous buffer. It is a small magnitude (0·4 %) in the whole expression. Since Randall and Failey's solubility correction [1927] for the variable ionic strength of the experimental solutions ($\mu = 0{\cdot}06$ to 0·23 according to p_H) is not greater than ± 3 % of α_{18}, it is not significant in these experiments.

When, during an experiment, a control manometer and flask containing 2·4 cc. water has been used to compensate readings of h for barometric fluctuations, the constant C becomes entirely independent of atmospheric pressure.

Observed pressure changes may therefore be converted directly into cc. of oxygen absorbed. In those cases where the manometers had been rapidly reset to cover pressure changes in excess of 250 mm., conterminous readings are simply totalled together, the point of resetting being appropriately indicated in the Figures and Tables. The oxygen uptake is then, after adjustment for y, plotted against time. Since the curve is approximately linear, the reaction may be defined directly by reading the appropriate absorption constant X/yt from the graph. Table II and Fig. 1 illustrate the procedure for a typical set of straightforward oxidations.

Table II. *Calculation of data. Variation of* k *with dialuric acid concentration.*

Citrate buffer; p_H 2·5.

m	0·0166 M		0·0125 M		0·0066 M		0·0063 M		0·0021 M	
V_s	2·4 cc.		2·4 cc.		1·2 cc.		2·4 cc.		2·4 cc.	
y	0·946		0·986		0·946		0·946		0·983	
Flask No.	(6)		(3)		(1)		(2)		(6)	
t (mins.)	h (mm.)	X/y (cc.)	h (mm.)	X/y (cc.)	h (mm.)	X/y (cc.)	h (mm.)	X/y (cc.)	h (mm.)	X/y (cc.)
2	−22	0·039	−18	0·027	−6	0·011	−12	0·021	−3	0·005
4	51	0·091	44	0·066	17	0·032	29	0·049	9	0·016
6	86	0·153	78	0·117	29	0·054	52	0·088	15	0·026
8	120	0·214	111	0·167	43	0·081	76	0·129	21	0·036
10	151	0·269	148	0·223	55	0·103	99	0·168	27	0·046
13	**200**	**0·356**	**200**	**0·302**	67	0·126	132	0·224	36	0·062
16	240	0·427	242	0·364	70	0·131	151	0·256	42	0·072
20	298	0·530	302	0·455	,,	,,	159	0·270	41	0·071
25	346	0·616	333	0·501	,,	,,	158	0·268	,,	,,
30	357	0·635	332	0·500	,,	,,	,,	,,	,,	,,
35	359	0·639	,,	,,	,,	,,	,,	,,	,,	,,
40	360	0·641	,,	,,	,,	,,	,,	,,	,,	,,
X/yt (mean)	0·029		0·026		0·012		0·019		0·005	
k	0·032		0·039		0·067		0·056		0·044	
log k	−1·50		−1·41		−1·17		−1·25		−1·36	

The constant X/yt, however, describes only a volume change, and will not reflect the molecular extent of the oxidation until it is converted into k, the degree of oxidation per minute $\left(\frac{X}{yt} \times \frac{1}{22{\cdot}4V_s nm}\right)$. Since $22{\cdot}4V_s nm$ is a constant in any one reaction, the calculation of k follows directly. k is utilised in its logarithmic form for the ultimate tabulation against temperature, concentration, p_H, *etc.*

It might be well to indicate that comparative tabulation of log k is quite valid whatever value is postulated for n. The expression $\frac{1000X}{V_s myt}$ (cc. oxygen per g.-mol. dialuric acid per min.) involves no assumption about the reaction,

but differs from k only in the constant, $22{,}400n$, which is characteristic of the reaction in general. In the comparative, as distinct from the absolute, evaluation of constants, no presumption is implicit as to the molecular proportions of the reaction.

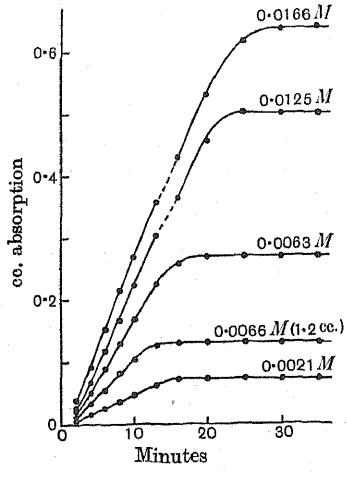


Fig. 1.

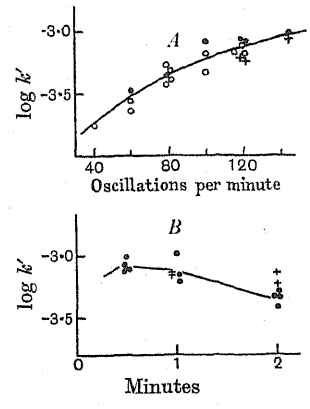


Fig. 3.

Fig. 1. Rate of absorption of oxygen. 2·4 cc. p_H 2·5. Citrate buffer. 18°.

Fig. 3. A. Effect of rate of oscillation. $k' = k\sqrt{m}\sqrt[n]{H^+}$. 2 min. readings. • p_H 7·9, + p_H 3·9 (1·2 cc., 0·0066 M. CO_2-free). o 0·005 and 0·01 M KCN (assorted). B. Effect of discontinuous oscillation. k' as above. 2·4cc. 0·0166 and 0·0125 M. CO_2-free. 120 oscillations per min. • p_H 7·9 and 9·0, + p_H 6·0.

Results.

Stoichiometric proportions.

If the autoxidation represents solely the production of alloxan, it should require two equivalents of oxidising agent, or 11·2 cc. oxygen per millimol. dialuric acid. In Fig. 2 the total oxygen absorbed experimentally is depicted, the included data being a selection, calculated as cc. oxygen per millimol. dialuric acid, from measurements which utilised not less than 0·03 millimol. dialuric acid in each experiment. The data differ in no significant fashion from those for the smaller amounts of dialuric acid. In those cases where the oxidation initiates CO_2 production, the CO_2 has been suitably removed. In Fig. 2 the maximum loss of reductant during temperature equilibration has also been inserted, after calculation from the rate of oxidation of dialuric acid at p_H 1·4 ($\log k = -1{\cdot}6$, see later). It is clear that, with the possible exception of the region p_H 3 to 4, the molecular proportion oxygen/dialuric acid is undoubtedly double the expected ratio. In other words, the reactants are combining in equimolecular proportions, so that $n = 1$. This value is embodied in subsequent calculation of k.

Since it was probable that the technique adopted by Hill [1930; 1931] had measured only the later stages of the reaction, Fig. 2 gives a comparison with a recalculation of Hill's data upon the same basis. The probability is confirmed.

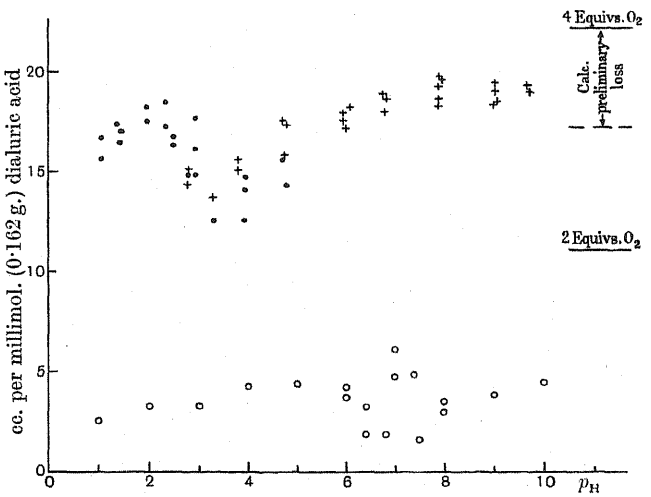


Fig. 2. Variation of total absorption with p_H. 2·4 cc. 0·0166 and 0·0125 *M*. • not CO_2-free, + CO_2-free, o data of Hill (10 cc., 0·018 and 0·0062 *M*).

Control of technique.

The rapidity of the reaction made it essential to determine the effect of varying technique upon the velocity measurements. In particular, such agitation of the reacting solutions was required that oxygen concentration would not be limited by its rate of diffusion. The rapidity of oscillation, therefore, was increased to the maximum speed of the apparatus by reducing the amplitude of swing to its limit (6 cm.). Unfortunately, this amplitude was still too great to permit the continuous reading of the manometers, and cessation of movement for a few seconds was ultimately necessary to obtain final readings after each approximate setting. Fig. 3 represents the definition of optimum conditions.

The data primarily represent alkaline oxidations, where it was expected that the speed of reaction would accentuate the possible inherent defects of technique. They represent a variety of values of p_H and m, and are therefore corrected to a common ordinate for convenient presentation by applying the conclusions of later sections. The separate portions of Fig. 3 represent (A) the conditioning of oxidation velocity by frequency of oscillation, and (B) the influence of discontinuous oscillation, interrupted at intervals of 2, 1 and 0·5 min., upon the observations. Since experiments, excepting those with added KCN, were conducted in the presence of a CO_2 absorbent, the efficiency of CO_2 absorption by 40 % NaOH on a cylinder of filter-paper must be known as a control. This has, however, already been examined by Dixon and Elliott [1930].

It is clear in section A of Fig. 3 that the rapid reaction is very considerably conditioned by the frequency of oscillation. It is probable that k' varies as some power of the frequency, and that, although an approximate maximum was achieved at the adopted frequency of 120 complete oscillations per minute (limited by the inertia of the apparatus) faster absorptions would obtain at greatly accelerated frequencies. The increase within realisable frequencies, however, would not greatly exceed the somewhat generous experimental error. The inserted curve expresses the arbitrary relation $k' = C'$ (frequency)$^{1\cdot33}$. It may be noted that the entirely distinctive reaction which occurs in the presence of 0·01 M and 0·005 M KCN, yet shows an effect of the degree of agitation which does not differ from the effect in the absence of KCN. In section B, it is apparent that interruption of oscillation becomes significant only for intervals of shorter duration than 0·5 min., and that 1 min. readings record the rapid reactions more closely than 2 min. readings. The variability is not appreciably greater than experimental error.

The velocity of autoxidation.

Fig. 4 depicts the gas volume change in a set of direct observations on dialuric acid solutions of varying p_H. The curves are clearly the resultant of two separable effects, namely, the effect of p_H on the rapidity of oxygen absorption (apparent more clearly from p_H 1·1 to 2·0), and the effect evident between p_H 4·5 and 11 of some opposing volume change which culminates

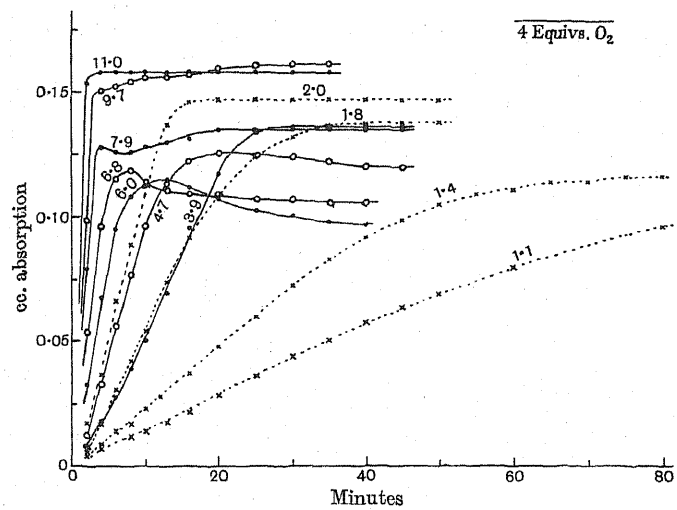


Fig. 4. Variation of absorption rate with p_H. 1·2 cc. 0·0066 M. Not CO_2-free. Universal buffer.

about p_H 6. The opposing reaction is slower in developing than is the oxidation, and is only able to reverse the volume change when oxidation is far advanced and somewhat slow. The effect is not large in extent, and would appear to have no appreciable influence on oxidation velocity during the

initial stage of the reaction. It is maximum between p_H 6 and 7. Fortunately it is completely eliminated by an effective CO_2 (or other volatile acid) absorbent, and under such conditions the measured volume change is, as has been noted in Fig. 2, closely similar to that of acid reactions and is in simple stoichiometric ratio with the dialuric acid present. All data now to be quoted, unless otherwise indicated, have been obtained in the presence of a CO_2 absorbent if alkaline to p_H 4·7. The further description of the opposing reaction is reserved for a subsequent paper.

Fig. 5 is an assembly of data obtained at varying p_H values and concentrations of dialuric acid, where each point represents a separate experimental

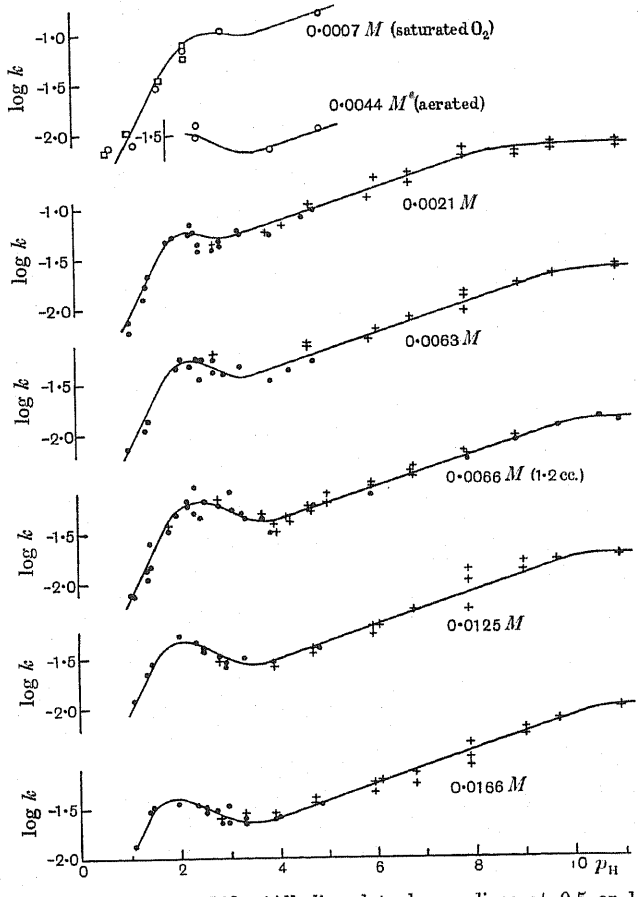


Fig. 5. log k:p_H curves. 2·4 cc. 18°. (Alkaline data by readings at 0·5 or 1 min. intervals.)
• not CO_2-free, + CO_2-free, o iodimetric data, □ $TiCl_3$ data.

curve of the type depicted in Figs. 1 and 4. The agreement is not as poor as might have been expected from the limited adaptability of the apparatus for rapid oxidations, and is quite sufficient to describe the situation with little uncertainty. It is seen that the effect of p_H is separable into three regions,

first, p_H 1 to 2, a region of rapidly increasing reaction velocity, next, p_H 2 to 4, one of uncertain interpretation which, significantly, corresponds with the incomplete oxidations of Fig. 2, and then finally, on the alkaline side of p_H 4, a region of steady increase of velocity as the 5th or 6th root of $[H^+]$, terminated by velocities too rapid for measurement. Log k in the inserted curves varies inversely as $[H^+]$ from p_H 1 to 2, and above p_H 4 inversely as $\sqrt[6]{[H^+]}$.

The fragmentary data included for 0·0007 *M* and 0·0044 *M* dialuric acid solutions are those already referred to [Richardson and Cannan, 1929] and are now included in full for reference. The 0·0007 *M* solutions were oxidised in a closed chamber by saturated solutions of oxygen (final conc. O_2, *ca.* 0·0011 *M*) and the reaction followed titrimetrically in two ways: firstly, by depressing the autoxidation of appropriate 25 cc. samples with excess HCl and immediate titration under nitrogen with 0·005 *M* iodine, and secondly the less satisfactory addition of excess $FeCl_3$ solution and back-titration with titanous chloride. The 0·0044 *M* solutions were oxidised by agitation by a stream of air and estimated iodimetrically. The data are not incompatible with the gasometric data now presented. In fact, the seemingly erratic behaviour and inconsequent relation to p_H that was then confusing is, between p_H 2 and 5, now found to be characteristic. Under both conditions of measurement, the reaction is found to have a linear velocity constant.

It is interesting finally to note that a stream of nitrogen produced, during a period of 45 min. at p_H 2·5, no change in the dialuric acid titre.

Effect of temperature.

The effect of temperature, as indicated by several experiments at 25° and 30° respectively, may be defined by the relation:

$$\log k_{t_2} = \log k_{t_1} + 0{\cdot}027\,(t_2 - t_1).$$

Fig. 6 (A) portrays the variation of the velocity of oxidation of 0·0066 *M* dialuric acid with temperature. The full curve is the curve at this concen-

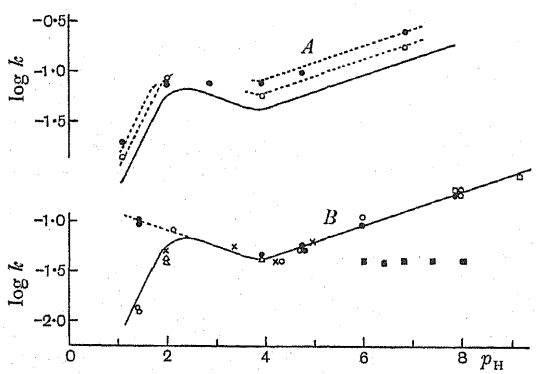


Fig. 6. A. Effect of temperature. o 25°, • 30°. B. Effect of added catalysts, o cyanide, • iron (data of Hill ■), □ glass rods, × barbituric acid, △ oxidised solution. log k:p_H curve (18°) from Fig. 5. 1·2 cc. 0·0066 *M*.

tration from Fig. 5, and the broken curves are the calculated curves for 25° and 30°, assuming the above relation to hold throughout the p_H range. It is noticeable that, whereas the p_H ranges 1 to 2 and 2 to 11 are separately differentiated with regard to catalysis or change of concentration, both p_H ranges are satisfactorily described by the one temperature coefficient.

Catalytic agents.

The roughly linear progress of oxidation is general evidence of a catalytic reaction embodying a constant concentration of catalyst. Further light upon the nature of this catalysis has been sought. In the first place, experiments were conducted at similar p_H values with different buffer solutions. Dilute HCl was used for the range p_H 0·7 to 1·7, citrate buffer was used for p_H 1·4 to 5, and universal buffer for p_H 2 to 11. No consistent differentiation was apparent, and no distinction is therefore made in Fig. 5 as to the buffers used in the respective experiments.

Although this in itself is probably sufficient evidence that catalysis was not due to the fortuitous presence of iron, definite observations on the catalytic effect of Fe and KCN on the oxidation are presented in Fig. 7 (A). It is clear

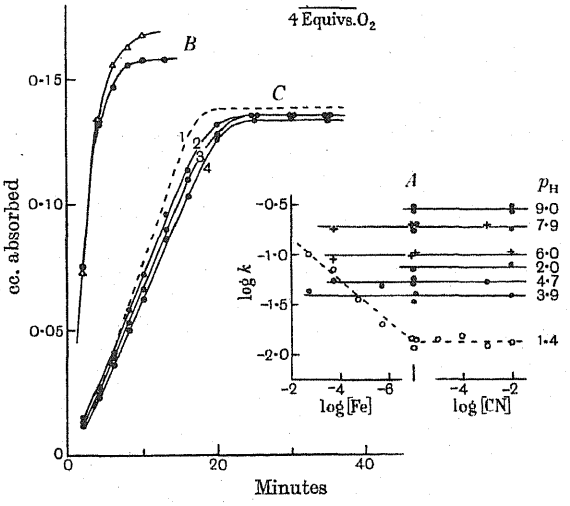
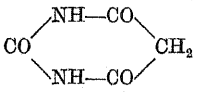


Fig. 7. A. Effect of iron and cyanide ions. • ○ not CO_2-free, + CO_2-free. B. Effect of glass surface. p_H 7·9. • plain, △ glass rods. C. Effect of constituent impurities. p_H 2·0. 1 plain, 2, 3 and 4, 0·005, 0·004 and 0·0016 *M* added oxidised solution, respectively. 1·2 cc. 0·0066 *M*.

from this that there is, except in acid solution, no catalytic effect of iron (or inhibitory effect of KCN) upon the reaction. This finding is especially noteworthy since the formation of the well-known blue iron complex in slightly alkaline solution (0·0016 *M* Fe, p_H 7·9) is thus found to be entirely without catalytic influence. In the case of acid solution, addition of cyanide was found to have no effect, whereas addition of iron led to steadily increasing velocities, defined in Fig. 7 (A) by the arbitrary straight line, $k = \sqrt[5]{[Fe]}$.

The total oxygen absorptions in the presence of smaller concentrations than 0·0001 *M* Fe or 0·001 *M* KCN, respectively, were of the usual extent. In the presence of 0·00016 *M* iron, however, oxygen absorptions were less than this, and with 0·0016 *M* iron were quite definitely one-half the usual extent even assuming partial oxidation by added iron (hence $n = 0{\cdot}5$). In alkaline Fe solution a certain confusion appears to arise from liberation of CO_2. Conversely, in the presence of 0·0083 *M* KCN, at p_H above 6, absorptions up to double the usual extent ($n = 2$) were obtained, although normal uptakes occurred at p_H values acid to 6.

Since the identity of velocity in different buffer preparations suggested the absence of catalytic impurity from the buffer, the dialuric acid preparation was next examined. The possibility of catalysis by a pyrimidine of similar structure was tested by the addition of barbituric acid,



possibly a contaminant by excessive reduction during preparation. No effect was observed, nor was barbituric acid itself oxidised. Next, a 0·02 *M* solution of dialuric acid in 0·001 *M* HCl was completely oxidised in a Warburg flask, and added, in final concentration 0·005 *M*, 0·004 *M*, and 0·0016 *M*, to fresh 0·0066 *M* dialuric acid solutions for oxidation experiments. Although the presumed catalyst must have been present in increased concentration at the initiation of these oxidations, the curves of Fig. 7 (C) show perfectly clearly that there has been no acceleration of the reaction.

The final possibility that catalysis was a surface phenomenon due to the glass container was tested by adding fine glass rods to increase the surface area of glass. Although the increase in wetted surface was from about 15 to about 30 cm.^{2}, no increased velocity was measured (Fig. 7 (B)).

In Fig. 6 (B), values of $\log k$ under different "catalytic" conditions in the oxidation of 0·0066 *M* dialuric acid are assembled, and compared with the mean curve for this concentration transcribed from Fig. 5.

The notable absence of catalysis induced extensively by any of the adopted procedures makes the linear order of reaction difficult to explain on any assumption that does not consider as a limiting factor the rate of permeation or of activation of oxygen. The fact that the one effective catalysis observed (Fe at p_H 1·3) has increased $\log k$ just so far as to align it with its more alkaline values while these values cannot themselves be increased, is itself rather significant in this respect. It appears that values greater than this cannot be obtained by catalytic acceleration of the oxidative reaction.

Effect of concentration.

A final indication as to the nature of the catalysis should be procurable from the relation of k to m. If z be g.-mol. of dialuric acid oxidised in time t, then when the reaction is limited by constant extraneous factors, such as

rate of activation of oxygen or catalysis by a constant impurity of the glass or buffer, the molar velocity constant should be $= z/t$. On the other hand, if the extraneous factors vary with m (*e.g.* catalysis by a constituent of the dialuric acid solution), the constant should be z/mt; this situation, however, has been rendered very improbable by Fig. 7 (C). Now k has actually been calculated as $x/t\,(= z/mt)$. k is therefore likely to vary, in accordance with the first situation, as $1/m$.

In Fig. 8, the comparison of velocity with concentration of dialuric acid is made by transcribing the curves of Fig. 5 upon a common ordinate. It is clear that at no p_H does $\log k$ vary as $-\log m$ in accordance with the requirements for constant catalytic activation. Indeed, between p_H 1 and 2, $\log k$ is very approximately non-variant with m, whereas on the alkaline side of p_H 4, it corresponds notably with the unforeseen relationship, $k = C''\,(1/\sqrt{m})$, indicated by the broken lines in Fig. 8.

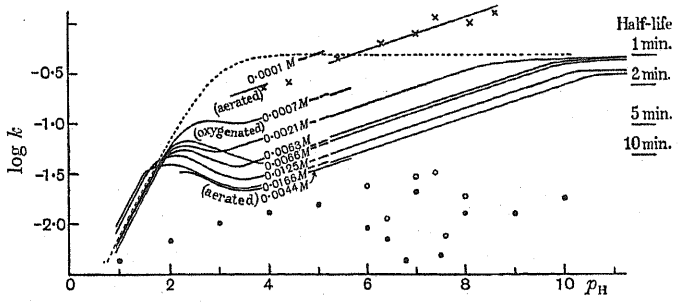


Fig. 8. Variation of $\log k$ with concentration of dialuric acid. •○ Data of Hill (0·018 and 0·0062 *M*, respectively). × Data of Clark, Cohen and Gibbs for leucomethylene blue.

It is interesting to observe the excellent agreement of the gasometric data with the data from titration of fully oxygenated mixtures. At the same time, however, the data obtained by titration of aerated solutions are definitely low, being included merely to show a general analogy between the curves. Yet it is a pointer to an unexpected correlation with tentative observations of Clark, Cohen and Gibbs [1925] on the autoxidation of leucomethylene blue by aeration; but even though their data as presented in Fig. 8 are convincingly close to the square root relation, it would be premature to press the analogy.

The data of Hill, replotted and recalculated according to the conventions of this paper, are included in Fig. 8 for reference. As is to be expected, the velocities measured fell considerably short of the velocities determined in the present work.

Of further interest in Fig. 8 is the possibility that the decline of the alkaline points from the $\sqrt[6]{[H^+]}$ relationship may be conditioned more by the acid dissociation of dialuric acid [Richardson and Cannan, 1929] than by the technical difficulty of measurement. The dotted line represents the maximum attainable velocities on the assumption that velocity varies directly as the

concentration of the ion dial.$^-$ and is definable by the actual measurements between p_H 1 and 2. In regions not greatly alkaline, however, it is presumed that this maximum velocity is not attained owing to its limitation by the external situation already mentioned. Although the half-life of dialuric acid is, as Fig. 8 indicates, so brief that uncertainty of measurement precludes any positive conclusion, it is at least clear that this interpretation would align itself remarkably with the tendency towards constant oxidation velocities in the more alkaline regions.

The autoxidation of alloxantin.

The experiments in which oxidised dialuric acid was added to solutions awaiting measurement (Fig. 7 (C)) were analogous, in effect, to measuring the autoxidation of alloxantin. Nevertheless, distinctive reaction velocities in these cases were not obtained. In seeking to confirm the observation, reactions were now conducted with freshly prepared alloxantin solutions, using an identical technique with that for dialuric acid. Owing, however, to the limited solubility of alloxantin, reaction mixtures more concentrated than 0·0055 M could not (with the exception of measurements in HCl) be obtained. Needless to say, alloxantin solutions of this strength remain only slightly associated, and are actually equivalent, as calculated from the association constant, to 0·0050 M solutions of dialuric acid. Fig. 9 depicts the results in the usual

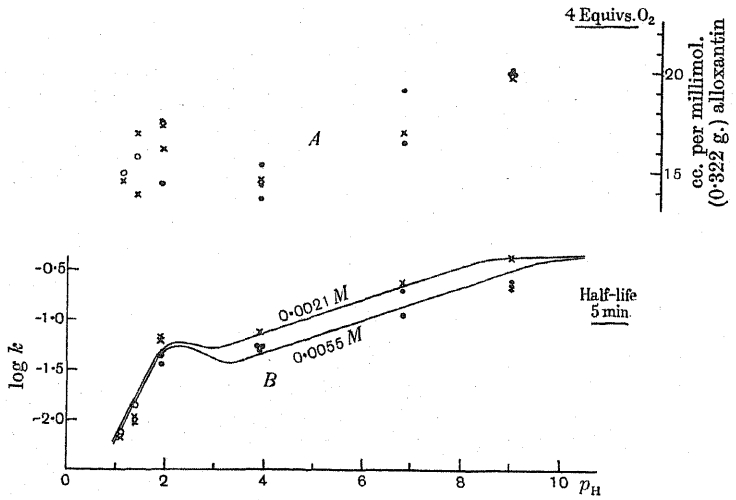


Fig. 9. Autoxidation of alloxantin. A. Total absorption. B. log k. 18°. ○ 0·0067M, • 0·0055M, × 0·0021M alloxantin.

fashion upon a basis of curves already defined, where, however, the curve for 0·0055 M solutions is calculated from the observed relation $k = C\,(1/\sqrt{m})$ (m = 0·005 M dialuric acid). The results clearly differ in no essential respect from those for dialuric acid. The oxygen uptakes were also of similar magnitude,

a selection of data for the more concentrated solutions being depicted in Fig. 9 upon a separate ordinate. Thunberg [1915], in two experiments on unbuffered saturated alloxantin solutions, measured uptakes in both cases in excess of two equivalents of oxygen, at rates such that the half-life of alloxantin was about 4 to 5 mins.

Alternative velocity constants.

The very striking variation of k as $1/\sqrt{m}$ over the p_H range 4 to 11 (Fig. 8) made it desirable to investigate the velocity equation

$$\frac{dz}{dt} = k_s' \sqrt{m - z},$$

from which by integration

$$k_s' = \frac{2\sqrt{m}}{t}\left(1 - \sqrt{1 - \frac{z}{m}}\right).$$

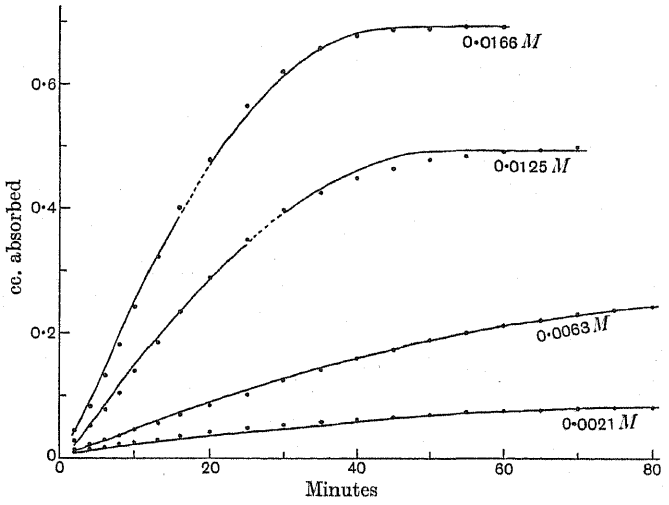


Fig. 10. Alternative velocity curves. 2·4 cc. p_H 1·4. HCl buffer. $\frac{dx}{dt} = k_s\sqrt{1-x}$.

Using x instead of z, these relations reduce to

$$\frac{dx}{dt} = k_s\sqrt{1-x} \text{ and } k_s = \frac{2}{t}(1 - \sqrt{1-x}),$$

in which k_s is non-variant with m. Fig. 10 shows that the expression fits the data for the slower and better defined oxidations with a fair degree of precision. Since it may be also calculated that

$$k = k_s\left(1 - \frac{k_s t}{4}\right),$$

or, provided the constants are calculated from early points such that kt is small compared with 4, that

$$k = k_s = \frac{k_s'}{\sqrt{m}},$$

variation of k_s with p_H is already satisfactorily defined in Fig. 5. These relations of k_s would derive chemically from the not impossible assumption that the

reaction is catalysed by both charges of the ion dial.$^{--}$, presumably by way of a double peroxide from two molecules of added oxygen.

Yet the formulation provides no better explanation of the region p_H 4 to 11 than does the simple linear reaction. In both cases, limitation of the velocity below the maximum formulated rates appears to operate. Again, whereas both velocity constants theoretically remain non-variant with concentration, the variation actually is as $1/\sqrt{m}$ in either case. In general, indeed, the relation

$$\frac{dx}{dt} = k_n (1 - x)^n$$

shows that k_n, for any order of reaction calculated in terms of x (which is non-variant with m), should itself be non-variant with m. Consequently, for the region p_H 4 to 11, where explanation in terms of linear constants has already been found inadequate, no explanation in terms of the ordinary velocity constants can be provided.

Discussion.

The fact that at p_H 7 oxidation was complete in considerably less than 10 mins. means that the experiments of Hill [1930; 1931] were conducted on solutions already considerably oxidised. This was initially suspected in a technique which exposed neutral solutions of dialuric acid to the atmosphere for an undetermined time before measurement, and which yielded thereafter plainly erratic total absorptions of oxygen. It was, however, an unfortunate circumstance that the few duplicates described in the second paper gave a general confirmation of the unduplicated findings of the first. In both cases, nevertheless, absorptions appear to have been very considerably below the maximum simply by reason of this initial oxidation (*cf.* Fig. 2).

It cannot be held therefore that alloxantin is the end-product of the reaction (an event unlikely by reason both of the large dissociation to its constituents in dilute solution, and of the existing evidence of Thunberg), since autoxidation has now been shown to proceed to completeness in definite stoichiometric proportions. The velocity equations of Hill are therefore in error. Again, for the reasons that the volume of solution was too large for efficient aeration (10 cc. in 33 cc. flasks), and that oxidation was in any case already far advanced, the actual velocities measured do not represent oxidation of dialuric acid under reproducible conditions, and can have no final significance (*cf.* Fig. 8). It is thus impossible to continue using this evidence to relate the oxidation-reduction in any way with the metabolic oxidation of cell material.

On the other hand, the oxidation of amino-acids in the presence of dialuric acid described in Hill's third paper [1932] is of considerable interest. But whether this is due merely to products of autoxidation, or to a definite reversible oxidation-reduction of dialuric acid has not yet been sufficiently defined. H_2O_2 is known to oxidise amino-acids in the given manner [Negelein,

1923; Breinl and Baudisch, 1907], while Hill discusses the similar reactions of alloxan. Unfortunately, however, the stoichiometry of either reaction near p_H 7 is not known. But it is conceivable that the end-products of oxidation of each equivalent of dialuric acid, may oxidise of themselves at least two equivalents of amino-acid. Therefore, since Hill measured greater values than this in only 2 of 11 experiments in reactions exceeding 5 hours, his evidence for catalysis by repeated oxidation-reduction of dialuric acid, remains inconclusive. In any event, it should be noted that alloxan as an individual constituent of cell material would have a half-life period not exceeding 2 mins. at any cell p_H alkaline to 6·5[1].

The possibility that both electrode potential and velocity of oxidation vary as a function of the dissociation constant of dialuric acid has been neither discounted nor more definitely manifested by the results of these experiments. But general correlation between potential and velocity has not been attained. The nature of the reaction appears to have been obscured by extraneous factors which the rapidity of oxidation allows to become operative.

Before considering these effects therefore the limitations of the technique should be briefly examined. Gasometric experiments have been conducted at 18°, the lowest temperature consistently available, and with air instead of oxygen as used previously, to minimise the rate of oxygen absorption. Nevertheless, the average absorption was at the rate of 1·5 to 3·0 cc. per hour, with a maximum of 12 to 15 cc. per hour. Dixon and Elliott [1930] state that under their conditions (32 cc. gas space, paraffin manometer fluid) measurements at rates limited to 1 cc. per hour are perfectly reliable, and they measure rates of 2·4 and 3·0 cc. per hour for cysteine and yeast without difficulty. Indeed, for rates of oxidation of pyrogallol of 18, 26 and 31 cc. per hour, only in the last case was there clearly discernible inconsistency. It is therefore probable that the rates measured herein are not greatly in error technically. Of this, the satisfactory concord between gasometric and titrimetric data is added evidence. This concordance of data is also evidence that aeration under the optimum conditions defined in Fig. 3 was actually equivalent to the complete oxygen saturation employed for the titrimetric data.

The extent of the error thought by Dixon and Elliott to arise from autoxidation of filter-paper under 40 % NaOH, does not appear to have been large under the present conditions, since no great discrepancy between oxygen absorption in the presence and absence of filter-paper-NaOH appears in Fig. 2.

As to stoichiometry, there is no doubt from Fig. 2 that, whereas only two electron equivalents are necessary for oxidation to alloxan, actually four equivalents of oxygen have reacted. Presumably this reaction is represented by: dialuric acid + O_2 = alloxan + H_2O_2. In the presence of iron, however, only two equivalents were absorbed; but since such conditions imply that H_2O_2

[1] Fig. 2, Section A of the previous publication [Richardson and Cannan, 1929] portrays, as is clear from Table V, the variation of log $10k$ with p_H, not of log k as indicated.

is not likely to be a stable end-product owing to its enhanced oxidising activity the reaction may be expected to proceed with one-half the oxygen absorption and no production of H_2O_2. It is interesting that the iodine titre of dialuric acid after oxygen absorption at p_H 5, 9 or 10 and acidification had entirely vanished. In such conditions, iodine does not react spontaneously with H_2O_2 [Liebhafsky, 1931].

The actual linear progress of the reaction characteristic between p_H 2 and 11 (*cf.* Figs. 2 and 4) is difficult to interpret. Not only did it occur where air-saturation was maintained by agitation, but even under conditions where the solution was pre-saturated with oxygen. It is therefore not a function of the rate of permeation of oxygen. Yet no change in the catalytic environment was able to point to its catalytic causation. It seems probable that the reaction is some obscure function of the rate of activation of oxygen, a conclusion supported by the positive temperature coefficient of the reaction, and by the unusual relations with $[H^+]$ and m. These relations may be defined by the equation $k' = k\sqrt{m}\sqrt[6]{[H^+]}$ or $k\sqrt{m}\sqrt[5]{[H^+]}$, where k' is a universal constant for the reaction between p_H 4 and 11. It is appropriate to recall that the tentative observations of Clark, Cohen and Gibbs [1925] described k for the autoxidation of leucomethylene blue also as a linear constant which varied as $\sqrt[5]{[H^+]}$, and that the magnitude of k is found in Fig. 8 to be very little removed from the $\sqrt{m}$ relationship described for dialuric acid.

Between p_H 1 and 2, the progress of oxidation is not so definitely linear, and may be described by an alternative velocity equation. k, by either derivation, is found to be non-variant with m and to vary inversely as $[H^+]$, relations which would follow directly from the chemical assumption that dialuric acid is oxidisable only in the form of its ion. It is interesting that addition of iron, which had no effect alkaline to p_H 2, between p_H 1 and 2 causes the reaction to become definitely linear and to proceed at velocities similar to those in more alkaline solution.

It is therefore suggested that in the autoxidation of dialuric acid under the observed conditions, the rate of activation of the reactants is of finite magnitude and varies between p_H 4 and 11 as $\frac{1}{\sqrt{m}\sqrt[6]{[H+]}}$; further, that where, by reason of p_H or of iron catalysis, the rate of reaction is greater than the rate of activation, the observed oxygen uptakes are conditioned in speed by the rate of activation. The velocity of the true reaction is therefore only observed where oxygen uptake proceeds slowly, a condition which is not fulfilled except between p_H 1 and 2.

Summary.

The rate of atmospheric oxidation of dialuric acid has been studied with reference to p_H, temperature, concentration of dialuric acid and influence of catalysts.

The reactants have been shown to combine in equimolecular proportions. In the presence of iron as catalyst, the proportion of oxygen was halved.

Alloxantin behaves as though completely dissociated to its components, dialuric acid and alloxan.

The suggestion that the oxidation-reduction has an important rôle in biological respiration has been rejected.

The author wishes to thank Prof. R. K. Cannan for his continued interest and encouragement during the course of this work.

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APPENDIX.

A selection of the data from which k has been calculated for 0·0166 M solutions is given below:

2·4 cc.; 18°.

p_H	Buffer	y	mm.³/min.	Total uptake (mm.³)	p_H	Buffer	y	mm.³/min.	Total uptake (mm.³)
1·1	HCl	0·97	12	625	4·8	Citr.	0·98	31	560
1·4	,,	0·95	27	695	6·0	U.B.	1·00	50	705
1·4	Citr.	0·95	29	660			0·96	54	735
2·0	U.B.	0·99	32	700	6·8	,,	0·98	59	760
2·5	Citr.	0·95	28	640			0·97	62	745
		0·99	27	650	7·9	,,	0·97	76	780
2·9	U.B.	0·97	21	590			0·97	90	775
3·3	Citr.	0·99	22	500			1·00	130	730
		0·99	26	550	9·0	,,	0·97	150	780
3·9	U.B.	0·97	24	600			1·00	180	740
		0·96	23	505	9·7	,,	0·99	220	765
4·7	,,	0·98	32	625	11·0	,,	0·99	275	755

CCXXXIV. FAT METABOLISM IN FISHES.

I. GENERAL SURVEY OF THE FATTY ACID COMPOSITION OF THE FATS OF A NUMBER OF FISHES, BOTH MARINE AND FRESH-WATER.

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(*Received October 20th, 1932.*)

The problems of fat metabolism in fishes appear in several ways to be somewhat different from those met with in the study of fat metabolism in either plants or mammals. The mixtures of fatty acids which go to form the fat of plants or mammals are much simpler than those of fishes, and it is difficult to find a reason for the special compositions of fish fats, with their numerous unsaturated components and high molecular weights. Two factors are probably of importance.

(*a*) Cold-blooded animals in general lay down a more unsaturated type of fat than warm-blooded ones. The effects of temperature on degree of unsaturation, as affecting both plant and animal depôt fats, are well established. This, however, will not explain the presence of fatty acids of such high molecular weight. It also does not account for the fact that the fats of marine mammals are mostly similar in general composition to those of the cold-blooded fishes.

Of the common marine species most are exclusively carnivorous, eating other fish. Thus their own fat composition will be decided largely by the type of fat in their food, since their carbohydrate intake is low, and fat synthesis from this source is not likely to be great. True, since protein can be transformed into carbohydrate, there is a possibility of fat being formed from protein, but the process seems an extravagant and wasteful one, and the writer is unaware of any evidence in support of this hypothesis. Thus it seems evident that if the smallest marine animals, which feed on marine flora, lay down these special fats, they will automatically bring about the storage of closely allied fats in the larger fish. It is therefore of interest to note that the marine flora contains poly-unsaturated fatty acids of high molecular weight similar to those found in the fat of fishes, although in very small amounts [Tsujimoto, 1925].

The number of species of fish whose fat has so far been quantitatively investigated is small, and it is to be noted that no single fresh-water species is

included. This is significant in view of the possibly different food supply of most fresh-water fish.

In 1930, Guha *et al.* [1930] published a paper in which was given a list of the quantitative compositions of the fatty acids of all the fish fats examined up to that date. This included only 13 species, and the authors pointed out that it was important that their survey should be greatly extended, in order to substantiate the biological group classifications which they made. This paper was followed by one from the writer [Lovern, 1930], in which further support was given to the previous authors' suggestions as to the peculiar relationships holding in the Elasmobranch family. Since that date the writer has not found opportunity to pursue this particular point further, but it is certainly one which will require a more detailed consideration. The only other quantitative data which the writer has observed are the partially quantitative analysis by Tsujimoto [1932] of a shark-liver oil of low iodine value, and the earlier work of Gill and Tucker [1930] on the composition of porpoise-jaw oil. The existence of such an oil as the last mentioned, of which the characteristic acid is *iso*valeric, illustrates one of the extraordinary complexities of fat metabolism in fishes.

The methods employed in the present work were practically identical with those given in detail in earlier papers [Guha *et al.*, 1930] and will not be described here. Considerations of space also preclude the giving of all the analytical data in detail, and only the final compositions will be included. One slight modification of method deserves mention. In the process of separating into liquid and solid acids by lead salt/alcohol, Banks [1932] found a much more complete separation when 0·5 % of acetic acid was added to the alcohol in the first precipitation. (This is, of course, always added of necessity in the subsequent recrystallisation of the solid lead salts.) The writer has used this new process with entire success.

In Table I are given the particulars of the oils, and in Tables II and III the percentage compositions of the fatty acid mixtures present in the new marine species examined. Table II is expressed in weight percentages, since this is the way in which previously recorded compositions have invariably been given, and Table III is expressed in molar percentages. In Table III is included an average of the previous group classifications, brought to molar percentages. The advantage of considering results from a molecular standpoint,

Table I. *Particulars of marine oils examined.*

Species	Description of oil
Haddock (*Gadus aeglefinus*)	Oil from the liver, the main storage depôt Iodine value 168·5 and unsaponifiable matter 0·7 %
Sprat (*Clupea sprattus*)	Oil from the head and body tissues, the main storage depôt Iodine value 150·7 and unsaponifiable matter 0·9 %
Angler (Monk) fish (*Lophius piscatorius*)	Oil from the liver, the main storage depôt Iodine value 162·3 and unsaponifiable matter 1·0 %
Halibut (*Hippoglossus vulgaris*)	Oil from the liver, one storage depôt Iodine value 120·0 and unsaponifiable matter 6·6 %

Table II. *Composition of mixed fatty acids (weight percentages) of oils of marine species.*

	Saturated			Unsaturated				
Species	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Haddock	4·3	14·1	0·3	0·5	12·4	30·5 (−2·6 H)	29·3 (−5·9 H)	8·6 (−7·3 H)
Sprat	6·0	18·7	0·9	0·1	16·2	29·0 (−2·9 H)	18·2 (−5·6 H)	10·9 (−7·1 H)
Angler fish	4·9	9·6	1·3	0·4	12·1	30·9 (−3·3 H)	24·9 (−5·9 H)	15·9 (−8·6 H)
Halibut	3·9	15·1	0·5	Nil	18·7	34·4 (−2·0 H)	13·8 (−5·5 H)	13·6 (−7·6 H)

Table III. *Composition of mixed fatty acids (molar percentages) of oils of marine species.*

	Saturated			Unsaturated				
Species	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Haddock	5·2	15·4	0·3	0·6	13·6	30·5 (−2·6 H)	27·1 (−5·9 H)	7·3 (−7·3 H)
Sprat	7·2	20·1	0·9	0·1	17·6	28·5 (−2·9 H)	16·5 (−5·6 H)	9·1 (−7·1 H)
Angler fish	6·0	10·6	1·3	0·5	13·5	31·2 (−3·3 H)	23·2 (−5·9 H)	13·7 (−8·6 H)
Halibut	4·7	16·3	0·5	Nil	20·4	34·0 (−2·0 H)	12·6 (−5·5 H)	11·5 (−7·6 H)
Gadidae*	6·5	12·0	Trace	Trace	15·5	29·0	25·5	11·0
Clupidae*	7	14·0	1·5	Nil	15·5	27·0	21·0	13·0
Balaenidae*	7·5	11·5	3·0	1·0	18·0	38·5	Variable	10·5

* Summarised from work of Guha *et al.* [1930]. Figures for Elasmobranch group, and sperm whale oils not included.

rather than one of mass has been pointed out by Hilditch and Sleightholme [1930].

The average unsaturation of the various groups has been expressed in terms of the number of hydrogen atoms required for saturation, as in previous papers. These values are in agreement with those previously recorded [Guha *et al.*, 1930] for the Gadidae and Clupidae oils. It is to be noted that each oil was a representative sample from a large number of fish, but even so, seasonal and other changes will account for minor fluctuations. In general, it will be seen that the oil from haddock-liver falls quite well into the Gadidae group, and that of the sprat into the Clupidae. These two groups are similar in composition. Angler fish-liver oil is similar to both. Halibut, however, is peculiar in that linoleic acid appears to be absent, and the proportions of acids of the C_{20} and C_{22} groups are nearly the same. Normally the C_{20} group greatly predominates. This seeming lack of linoleic acid will be referred to again. Conjointly with the reduced amount of acids of the C_{20} group it will account for the low iodine value of halibut-liver oil.

Up to the present five oils from fresh-water fish (4 species) have been examined in detail, and in Tables IV, V and VI are given the particulars and the compositions.

Table IV. *Particulars of oils from fresh-water fish.*

Species	Description of oil
Carp (*Cyprinus carpio*)	Oil from head and body tissues, the main fat depôt Iodine value of oil = 122·9 and unsaponifiable matter = 3·5 %
Pike (*Esox lucius*)	(*a*) Oil from head and body tissues, one of two main fat stores Iodine value of oil = 145·9 and unsaponifiable matter = 4·0 % (*b*) Oil from the mesentery, the other main fat depôt Iodine value of oil = 134·2 and unsaponifiable matter = 0·1 %
Pollan (*Coregonus pollan*)	Oil from head and body tissues, the main fat depôt Iodine value of oil = 136·8 and unsaponifiable matter = 1·5 %
Perch (*Perca fluviatilis*)	Oil from head and body tissues, the main fat depôt Iodine value of oil = 131·7 and unsaponifiable matter = 6·0 %

Table V. *Composition of mixed fatty acids (weight percentages) of oils of fresh-water species.*

	Saturated			Unsaturated				
Species	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Carp	3·7	14·6	1·9	1·0	17·8	45·8 (−3·2 H)	15·2 (−6·9 H)	Nil
Pike (body)	4·7	13·2	0·5	0·8	20·8	38·4 (−2·8 H)	15·3 (−7·5 H)	6·3 (−7·5 H)
Pike (mesentery)	2·9	15·0	Trace	0·5	20·2	42·4 (−3·4 H)	15·1 (−6·7 H)	3·9 (−8·0 H)
Pollan	2·9	14·3	1·9	1·5	19·8	40·0 (−3·2 H)	13·5 (−7·4 H)	6·1 (−9·1 H)
Perch	3·5	12·5	2·0	1·1	19·4	40·5 (−3·2 H)	13·8 (−6·8 H)	7·1 (−9·2 H)

Table VI. *Composition of mixed fatty acids (molar percentages) of oils of fresh-water species.*

	Saturated			Unsaturated				
Species	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Carp	4·4	15·5	1·8	1·2	19·0	44·5 (−3·2 H)	13·6 (−6·9 H)	Nil
Pike (body)	5·6	14·1	0·5	1·0	22·4	37·4 (−3·0 H)	13·8 (−7·5 H)	5·2 (−7·5 H)
Pike (mesentery)	3·4	16·0	Trace	0·6	21·7	41·5 (−3·4 H)	13·6 (−6·7 H)	3·2 (−8·0 H)
Pollan	3·4	15·5	1·8	1·8	21·2	39·0 (−3·2 H)	12·2 (−7·4 H)	5·1 (−9·1 H)
Perch	4·2	13·4	1·9	1·3	20·9	39·8 (−3·2 H)	12·5 (−6·8 H)	6·0 (−9·2 H)

Comparing these data with those for the marine oils (Table III), we find:

(*a*) The oleic-linoleic acid group percentage consistently higher in fresh-water fish fats.

(*b*) The C_{20} and C_{22} groups present in considerably smaller amounts in fresh-water fish fats.

(*c*) Palmitoleic acid is a more important component of all the fresh-water fats than of the marine ones, with the exception of halibut-liver oil, which is unique.

(*d*) Otherwise, the fresh-water and marine oils are similar, in that in each class the average unsaturation of the various groups is of the same order, and the general proportions of the fatty acid mixture are the same, *viz.* small quantities of myristic acid, with traces only of myristoleic acid; considerably greater quantities of palmitic and palmitoleic acids (in comparable amounts); traces only of stearic acid, with oleic and linoleic acids as one of the main components of the oils; the C_{20} group, having no saturated member, also a major constituent, and the C_{22} group, still more unsaturated, in smaller amount.

Banks and Hilditch [1932], working on the body fats of the pig, have shown that there is a balance between stearic and oleic acids, tending to keep the total amount of C_{18} acid approximately constant, however much either component varies. Stearic acid is not a major component of fish oils, but palmitic is, and it is of interest to compare the total group percentages (molar) for all the oils described.

Table VII. *Total groups of acids. Molar percentages.*

Species	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Marine:					
Haddock	5·8	29·0	30·8	27·1	7·3
Sprat	7·3	37·7	29·4	16·5	9·1
Angler fish	6·5	24·1	32·5	23·2	13·7
Halibut	4·7	36·7	34·5	12·6	11·5
Gadidae (average)	6·5	27·5	29·0	25·5	11·0
Clupidae (average)	6·0	27·2	28·7	22·5	15·5
Fresh-water:					
Carp	5·6	34·5	46·3	13·6	Nil
Pike (body)	6·6	36·5	37·9	13·8	5·2
Pike (mesentery)	4·0	37·7	41·5	13·6	3·2
Pollan	5·2	36·7	40·8	12·2	5·1
Perch	5·5	34·5	41·7	12·5	6·0

It will be noticed that there is a tendency towards an approximate constancy of the amount of C_{16} acids in the fresh-water fish, but no constancy in the marine species. This may, however, be fortuitous with so few samples. It may be significant that the two marine fats with total C_{16} acids of 37 % (sprat and halibut) are also comparable with the fresh-water fats in having a low C_{20} content.

In the paper of Guha *et al.* [1930] it is stated that the poly-unsaturated acids of high molecular weight characteristic of fish oils are evidently marine in origin. This cannot now be accepted unreservedly—they appear to be characteristic of aquatic life (including marine algae, *etc.*) of all kinds, including fresh-water. The diet of fresh-water fish is perhaps different from that of marine species. Certainly many are purely carnivorous, but in many cases they eat such species as the young of small birds (the particular pike examined,

for instance, all had small wild-ducklings in their stomachs), frogs, and even mice, *etc.* Others are herbivorous. It has not been shown whether the small crustacea, worms, *etc.*, and the flora of fresh-water contain these typical fish oil acids. It is hoped later to investigate this point.

The high content of oleic and linoleic acids in the fats from the fresh-water fish is paralleled in the marine mammalia (Balaenidae), but the parallelism does not extend to the other groups of acids. However, in all land mammals, the oleic-linoleic acid group is the only unsaturated one present, and hence we may say that the fats from fresh-water fish, as well as those from marine mammals, are of a type intermediate between the fats from marine and terrestrial animals. Differences of food might account for this, if the natural tendency of all fish was to lay down fats of the marine type. It is hoped later to conduct controlled feeding experiments on both fresh-water and marine species to decide this point, but the technical difficulties are considerable. Two other factors must be borne in mind. The average temperature of fresh-water ponds and rivers, especially shallow ones, is likely to be higher than that at the bottom of the sea. Also, the salinity of sea-water may have some effect, although the writer does not consider this probable. It can fairly easily be determined with species which can be kept in both fresh and salt water, *e.g.* eels. It is well not to underestimate the effects of environmental and seasonal differences, as contrasted with the effects of food differences, since the work of Hilditch and Sleightholme [1930] has shown that, in the case of butter fats at all events, the former may far outweigh the latter.

The apparent lack of linoleic acid in the case of halibut-liver oil requires further notice. It is now almost certain that mammals, for example, cannot synthesise linoleic or linolenic acids from carbohydrate, and that when deprived of them in their diet they suffer in health. Administration of either acid will alleviate the trouble [Evans and Lepkovsky, 1932; Burr, Burr and Miller, 1932]. Earlier work on different lines by Ellis and Harkins [1925] on the composition of pig fats lends decided support to this view, that all the linoleic acid present has come directly from ingested linoleic acid. The results of Hilditch and his collaborators [Banks and Hilditch, 1932; Bhattacharya and Hilditch, 1931] on pig fats are also in accordance with this theory. There is no evidence as to whether or not linoleic acid is essential to the well-being of fishes, but it is significant to note that the highly unsaturated arachidonic acid did not alleviate the symptoms in the experiments of Burr, Burr and Miller [1932]. Either linoleic acid is not essential to the halibut (it must be remembered that the diet of the halibut will include linoleic acid), and is hence readily destroyed by oxidation, or else it was present, but in traces too small for detection by the analytical methods employed. There is a third possibility. The liver is not the only fat store in the halibut, and there are quite considerable amounts of fat deposited just beneath the skin. It would be necessary to analyse fat from every single depôt in the fish before it could be said that linoleic acid was definitely absent.

Nevertheless, the composition of halibut-liver oil is unusual in this respect, and also in its low content of acids of the C_{20} series. The writer is frequently examining samples of halibut-liver oil in connection with another piece of work, and has found that the iodine value is invariably low, and of the order of 120, as with the sample analysed. Thus the possibility of it being a "freak" sample is rather remote.

One other point is worthy of mention. It was indicated in the earlier work of Guha *et al.* [1930] that the widely varying extents to which the highly unsaturated esters tended to polymerise on heating suggested that the structures of the acids were not necessarily similar in the different oils. The writer has observed the same marked peculiarities in the present work, and also a possibly associated fact, that with some oils the highly unsaturated esters which distil over have a deep golden-yellow colour, whilst with others the distillates are almost colourless. This is quite irrespective of the iodine value of the fractions, or of the colours of the original oils, and is not due to traces of pigment. This point seems worthy of further investigation, and is being followed up.

Summary.

A start has been made on a series of investigations into the peculiar problems of fat metabolism in fishes. The work of Guha *et al.* [1930] has been extended as a preliminary to more detailed work, and the present paper includes data for a number of oils, including five from fresh-water fish.

Significant differences in fatty acid composition are apparent between the oils from fresh-water fish on the one hand, and from marine species on the other. The most noticeable are the increased proportions of oleic and linoleic acids, and also of palmitoleic acid, in the fresh-water fish oils, and the reduced amounts of acids of the C_{20} and C_{22} series. Possible reasons for this are mentioned.

The composition of a sample of halibut-liver oil, representative of many fish, has been shown to be unique in respect of an apparent entire lack of linoleic acid, and of the low ratio of C_{20} to C_{22} acids. The significance of this absence of linoleic acid is discussed.

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CCXXXV. FAT METABOLISM IN FISHES.

II. THE PERITONEAL, PANCREATIC AND LIVER FATS OF THE STURGEON (*ACIPENSER STURIO*).

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The data presented in the previous paper [1932] were all connected with fats which represented the main depôt fat of each species investigated, and, with the exception of the pike fats, the fat from only one depôt was used in each species. The quantity of any particular fat employed for a quantitative analysis of the component fatty acids should preferably be of the order of 200 g. and with less than 150 g. the accuracy will begin to fall off. This means that in many cases only the main fat depôts can be dealt with. However, with very large fish, there may be enough fat available from a number of sources. The writer was fortunate to obtain the whole of the viscera and a large piece of the peritoneal fat (the main fat store in the case of this fish) of a large sturgeon (*Acipenser sturio*) measuring over 11 ft. in length. The liver was not exceptionally rich in fat, but as it weighed 14 lbs., there was an ample supply of liver fat for analysis. The pancreas was loaded with fat, and a large sample was obtained. No opportunity was afforded for the examination of the flesh oil, but it was evident that the flesh itself was not of an oily nature, and the peritoneal fat may be taken as being the main deposit of stored fat.

The compositions of the fatty acids from these three oils are given in Tables I and II, and as before, the average unsaturation of each group is expressed in terms of deficiency in hydrogen.

Consideration of these figures reveals several interesting points. The writer was surprised to find that the liver fat was apparently no more unsaturated

Table I. *Composition of fatty acids in weight percentages.*

		Saturated			Unsaturated				
Oil from	Iodine value	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Peritoneal cavity	126·5	7·1	14·0	0·8	0·6	23·8	35·8 (−2·9 H)	12·1 (−7·4 H)	5·8 (−8·6 H)
Pancreas	119·6	4·5	16·4	1·1	Nil	21·4	36·7 (−2·9 H)	14·5 (−6·8 H)	5·4 (−9·1 H)
Liver	125	3·0	19·2	Nil	Nil	19·5	39·6 (−2·7 H)	11·8 (−7·1 H)	6·9 (−10·0 H)

Table II. *Composition of fatty acids in molar percentages.*

Oil from	Iodine value	Saturated			Unsaturated				
		C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Peritoneal cavity	126·5	8·4	14·7	0·8	0·7	25·3	34·5 (−2·9 H)	10·8 (−7·4 H)	4·8 (−8·6 H)
Pancreas	119·6	5·3	17·4	1·1	Nil	22·9	35·7 (−2·9 H)	13·1 (−6·8 H)	4·5 (−9·1 H)
Liver	125	3·6	20·5	Nil	Nil	20·9	38·6 (−2·7 H)	10·6 (−7·1 H)	5·8 (−10·0 H)

than the main depôt fat, as indicated by the iodine values, and the more detailed analysis bears this out. This is contrary to what obtains with mammals, and from the figures given above it seems difficult to imagine a process of desaturation going on in the liver of the sturgeon. The degrees of average unsaturation of the various groups of acids in the three oils are indeed so much of the same order, that the differences might be considered as insignificant. If, however, they are taken into account, they seem to be against the theory that the liver fatty acids (it being remembered that throughout it is glyceride fatty acids that are referred to, and not phospholipin fatty acids) are more unsaturated than the fatty acids found in other fat depôts in the fish. The only grouping which is definitely more unsaturated in the liver fat than in the peritoneal fat is the C_{22} group, and for purposes of metabolism one would have thought that the acids in this group were unsaturated enough. It must not be forgotten, however, that associated with the highly unsaturated C_{22} acids there is usually some monoethylenic acid and it may be that this is further desaturated. The evidence of the other groups is against this supposition however. The C_{20} group is definitely most unsaturated of all in the storage fat, whilst the oleic-linoleic group is least unsaturated in the liver. But it is when we come to the palmitic and palmitoleic ratios that the figures seem more definite. Whilst the palmitic acid figure steadily rises from the peritoneal fat to the liver fat (*i.e.* from stored fat to fat probably taking part in active metabolism), the palmitoleic acid figure steadily falls. The total C_{16} grouping is remarkably constant, the figures being 40·0, 40·3 and 41·4 respectively for the peritoneal, pancreatic and liver fats. This steadiness between the main saturated component and its unsaturated derivative seems to the writer in full accordance with the results of Banks and Hilditch [1931; 1932] for pig fats, from which they conclude that there is inter-convertibility between saturated and unsaturated homologues. Their figures lead them to the opinion that the process is one of saturation to meet the normal body storage fat requirements, rather than one of desaturation. The present data throw no light on this interesting point, unfortunately, but they do support the argument that there is conversion of one acid into the other.

It seems of value to determine whether this relative saturation of the liver fat of the sturgeon is paralleled in the marine mammals. The writer has secured a medium-sized porpoise, and hopes to be able to pursue this point by full

analyses of the fats from various organs as well as the blubber. It must be borne in mind, of course, that fish fats are of an essentially different, and more reactive, nature than the body fats of land mammals, and their metabolism may be more direct.

There is another remarkable constancy to be observed. The total saturated acid percentages (sum of myristic, palmitic and stearic acid figures) are 23·9, 23·8 and 24·1 % respectively for the peritoneal, pancreatic and liver fats, in spite of quite considerable variations in the constituent acids—myristic from 3·6 to 8·4 %, palmitic from 14·7 to 20·5 % and stearic from 0 to 1·1 %. This strongly suggests that the three saturated acids are of equal value to the fish, and that myristic acid can replace palmitic acid functionally to a considerable extent. The lower percentage of palmitic acid would be compensated for by conversion of the excess palmitic into palmitoleic acid, thus keeping the total C_{16} acid percentage approximately constant. The net result, therefore, is strikingly in agreement with the conclusions of Banks and Hilditch, and we may picture the scheme of things in the sturgeon as follows.

The fat in all deposits requires an approximately constant relationship between the amounts of saturated and unsaturated acids present, *viz.* 24 % of saturated and 76 % of unsaturated. The exact significance of the variations in the percentages of the respective components within the saturated group is not clear, but it seems not improbable that the greater reactivity of myristic acid as compared with palmitic, and the different conditions of oxidation, *etc.*, in the various organs may have something to do with it. At any rate, we may suppose that if myristic acid is being destroyed at an excessive rate, palmitoleic acid will be saturated to increase the palmitic percentage, and so keep the ratio between saturated and unsaturated acids constant. On the other hand, we must equally suppose that if myristic acid is entering a depôt in unusually large amounts, some palmitic acid will be desaturated to palmitoleic acid. Feeding experiments will be necessary to settle this point. The rôle of stearic acid may well be similar to that of palmitic acid, but in a minor degree. The writer would have been disinclined to draw such far-reaching conclusions from the data of one fish, had not the work of Banks and Hilditch afforded such strong support.

Naturally, such relationships would not be expected to hold amongst all the oils from various marine species, as there seems no reason why all gadoid fish, for instance, should require the same ratios. It may be fortuitous that for all the fresh-water fish oils given in the previous paper, the sum of the myristic, palmitic and stearic acid percentages approximates to 20.

Two further points of interest are the following:

(*a*) In respect of the high content of palmitoleic acid, and the constant proportion of total C_{16} acids, these oils resemble those of the fresh-water fish rather than those of marine species.

(*b*) The high content of oleic and linoleic acids, together with the reduced amounts of C_{20} and C_{22} acids, is also suggestive of a fresh-water fish.

Now the sturgeon is a fish which inhabits both fresh water and salt. The particular specimen from which these fats came had been caught in the North Sea, but all sturgeon ascend the large Continental rivers to spawn, and moreover do most of their feeding in fresh water. Thus it is hardly surprising to find that the fats are of the same type as those of a true fresh-water fish, and in fact the sturgeon figures are additional support for the genuineness of the differences pointed out in the previous paper between fats from marine species on the one hand and fresh-water species on the other.

Summary.

The fats from the peritoneal cavity (main storage fat), pancreas and liver of a large sturgeon have been analysed as regards their component fatty acids. The results do not support the theory of desaturation in the liver, in the case of this particular fish. It is hoped further to investigate this point in respect of a marine mammal.

The data do, however, agree with the view that there is an inter-relationship between the main saturated acid component which has an unsaturated derivative—stearic and oleic acids in the case of pigs, *etc.*, and palmitic and palmitoleic acids in the case of fish. There is no evidence, however, as to the seat of these transformations.

There is, moreover, distinct evidence that a definite relationship is maintained between the amount of total saturated acids present, and total unsaturated acids—in the case of the sturgeon the values being 24 % of saturated and 76 % of unsaturated acids. Thus the other main saturated component, myristic acid, must enter into the scheme, and a relationship is adduced between myristic, palmitic and palmitoleic acids.

The results support in every respect the provisional conclusions drawn in the previous paper as to the characteristic differences between the fats of marine fish and fresh-water fish.

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CCXXXVI. THE HYDROLYSIS OF CASEINOGEN BY PEPSIN AND BY TRYPSIN-KINASE.

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During the past few years the existence of an integral molecule of caseinogen has been questioned. Linderstrøm-Lang [1928] has extracted caseinogen with a variety of warm (60°) solvents (chiefly 60 % alcohol with or without $N/1000$ HCl and dilute HCl-NaCl solutions) and has been able to separate the protein into various fractions which differ in a number of respects, notably in their phosphorus contents. The interpretation of these experiments is difficult, for no information has, as yet, been obtained as to whether the reaction between solvent and substrate is physical or chemical. Linderstrøm-Lang, however, believes that caseinogen is heteromolecular, that it is in reality a loose physical association of several proteins of different phosphorus contents which, in all the processes to which caseinogen is ordinarily subjected, form a co-precipitation system.

Whatever interpretation be adopted, whether the various fractions are to be considered as existing chemically bound to give an integral caseinogen molecule, or whether they are held together in a species of loose physical association, it would render untenable the idea that all the phosphorus of caseinogen is concentrated in one "prosthetic" group. This is difficult to reconcile with the finding of Rimington [1927, 1] that, by the action of dilute alkali on caseinogen, he could obtain a protein containing 11 to 13 % nitrogen and only slight traces of phosphorus. Rimington [1927, 2] was also able to isolate from tryptic digests of caseinogen an enneapeptide triphosphoric ester (phosphopeptone) in amount representing about 50 % of the original caseinogen phosphorus. In terms of the Linderstrøm-Lang hypothesis, caseinogen must then be a loose physical association of large molecules, which, when subjected to the disruptive action of alkali, loses its phosphorus and still remains a co-precipitation system—the "dephosphorised caseinogen" of Rimington. Such a requirement imposes a considerable strain on the "loose association" hypothesis.

Rimington and Kay [1926] have investigated the action of pepsin and trypsin[1]

[1] Trypsin, in our paper, is used to denote the mixture of enzymes usually found in pancreatic extracts. This consists, according to the nomenclature of Waldschmidt-Leitz [1929], of trypsin (inactivated) trypsin-kinase (activated) and erepsin. Of these only trypsin-kinase has any action on caseinogen, although the others can hydrolyse its lower degradation products.

on caseinogen. They confirmed the findings of previous workers that during peptic digestion an insoluble product is formed (para- or pseudo-nuclein) which contains a large proportion of the caseinogen phosphorus. They also showed that, at the end of 9 days' digestion with pepsin, no measurable amount of free phosphate was obtained, though all the caseinogen phosphorus was now in a form not precipitated by trichloroacetic acid. With trypsin, it was found that there was a fairly rapid liberation of organically-bound acid-soluble phosphorus with accompanying slow liberation of inorganic phosphorus. The liberation of amino-acid nitrogen was followed simultaneously with that of acid-soluble phosphorus but no information was obtained regarding the partition of phosphorus between the various products formed at different stages of the digestion. Linderstrøm-Lang and his co-workers have recently [Holter, Linderstrøm-Lang and Funder, 1932] studied, in some detail, the peptic digestion of caseinogen and of the fractions obtained by Linderstrøm-Lang [1928]. They found that the precipitates (paranuclein) formed in each case had almost identical N/P ratios but that these values were quite different from that obtained by Rimington for his phosphopeptone. Linderstrøm-Lang suggests that paranuclein may be formed during tryptic digestion and that it may be the precursor of Rimington's phosphopeptone. In this connection it must be remembered that the solubility of paranuclein may be a function of p_H and its failure to precipitate during tryptic digestion may be due to the alkaline reaction of the digestion solution. None of the work quoted can therefore be taken to indicate that the action of pepsin on caseinogen is fundamentally different from that of trypsin. Waldschmidt-Leitz and Simons [1926] have demonstrated that both pepsin and trypsin-kinase act on caseinogen by the rupture of amide-linkages, *i.e.* for every amino-group set free a carboxyl is liberated. To demonstrate whether or not the same amide-linkage of caseinogen is attacked by both enzymes requires other methods of investigation than those already reviewed.

With regard to the initial attack of trypsin on caseinogen Northrop [1924] has made certain assumptions on which he has built a method for investigating the kinetics of the action of the enzyme. Northrop wished to study the effect of increase of caseinogen concentration on the rate of disappearance of caseinogen and he was forced to employ a method which, at any instant, would separate the protein from its cleavage products and so allow the amount of the former to be estimated. To this end he added trichloroacetic acid to the digestion solution at successive intervals of time and assumed that the precipitate represented unchanged caseinogen. He makes the statement that the first step in the action of trypsin, *i.e.* the hydrolysis of the caseinogen molecule itself, results in the formation of products completely soluble in trichloroacetic acid. If Northrop's conception of the initial step of the hydrolysis of caseinogen be correct, we should expect that, at any instant during digestion, the N/P ratio of a trichloroacetic acid filtrate should be identical with that of the original caseinogen. Preliminary experiments carried out by

us showed that this was by no means the case. Moreover we have found significant differences between the behaviour of pepsin and trypsin so far as the course of the acid-soluble N/P ratio is concerned, differences which appeared to indicate distinct differences in the mode of attack of the two enzymes. Accordingly, in the present paper, we report experiments designed to follow the liberation from caseinogen by the action of pepsin and of trypsin-kinase of nitrogen and phosphorus compounds soluble in trichloroacetic acid.

It would appear that no disadvantage is attached to the use of commercial pepsin preparations in view of their freedom from other proteolytic enzymes and accordingly we have employed B.D.H. pepsin (granular) in these studies. The use of commercial trypsin, however, appears to require some justification. Willstätter *et al.* [1926] have shown that, of the pancreatic proteases, only trypsin-kinase has any action on caseinogen. The others have no action on any molecule of magnitude greater than polypeptides and the latter being soluble in trichloroacetic acid the action of the peptidases will be confined entirely to the acid-soluble material resulting from the hydrolysis of caseinogen and its acid-insoluble degradation products. The change from acid-insoluble to acid-soluble will therefore be due entirely to the action of trypsin-kinase. We have used, in these experiments, an Allen and Hanbury's trypsin preparation without further purification, and we shall refer to the action of this preparation as being that of trypsin-kinase.

In view of the doubt expressed regarding the constancy of the composition of caseinogen, it is well to refer to the source of our specimen. It was prepared from milk by the improved electrodialysis method of Van Slyke [1923] and was a pure white product of N/P ratio 20·0 (caseinogen "nach Hammarsten" has N/P 18·6 while the purest specimen obtained by Van Slyke had N/P 22·2). It contained a negligible quantity of acid-soluble nitrogen and no acid-soluble phosphorus.

Experimental.

Hydrolysis by trypsin-kinase.

Caseinogen solution. This was made up fresh before each experiment and of twice the concentration desired in the digestion solution. The caseinogen was rubbed to a paste with distilled water in a mortar and dilute NaOH added slowly with stirring (0·8 cc. *N* NaOH per g. caseinogen). When all the alkali had been added the liquid was centrifuged and the clear solution removed from a slight sediment and diluted to 100 cc. with distilled water. In this way a solution of p_H slightly greater than 7·0 was obtained.

Enzyme solution. This was a 1 % filtered solution of Allen and Hanbury's trypsin and was made up fresh for each experiment. The amount of enzyme required to achieve the degree of hydrolysis desired in a given time was determined by preliminary experiment and was obtained by dilution of the 1 % solution.

Buffer solution. The optimum range of trypsin activity is p_H 7·8–8·7 [Waldschmidt-Leitz, 1923–24]. During digestion of caseinogen there is a very considerable shift of p_H towards the acid side and with the higher protein concentrations buffering is a matter of some difficulty. In our experiments the p_H has been followed throughout the course of the digestion (hydrogen electrode) and the maximum shift found was about 0·3 p_H. As the digestion solutions were always initially buffered to a p_H near to the upper limit of the optimum range it can be said that at no time, during the course of the digestion, was the enzyme subjected to a p_H below the lower limit of the optimum. The buffer used was the KCl-borate mixture (p_H 8·8) of Clark and Lubs [Clark, 1928] and was made up in a concentrated solution. (Clark and Lubs buffer was obtained by diluting 4 cc. of this concentrated solution to 10 cc.)

Procedure. A series of stoppered tubes was made up, each containing 5 cc. caseinogen solution and 4 cc. concentrated buffer solution. The tubes were placed in the thermostat and after they had attained the desired temperature (37°) 1 cc. diluted enzyme solution was added to each. At 10 or 15 minute intervals a tube was withdrawn and 10 cc. 10 % trichloroacetic acid were added. After shaking, the contents were immediately filtered and acid-soluble nitrogen (micro-Kjeldahl) and phosphorus [Fiske and Subbarow, 1925] determined on aliquot portions of the filtrate. The total nitrogen and phosphorus and initial acid-soluble nitrogen and phosphorus contents were determined on enzyme-containing solutions which had not been placed in the thermostat. Controls were run by incubating tubes containing distilled water instead of enzyme solution. The changes followed by us were found to be due wholly to the presence of the enzyme as the buffer alone did not produce any hydrolysis.

Results. The amounts of acid-soluble nitrogen and phosphorus liberated at successive intervals of time with four different concentrations of caseinogen

Table I A. *Trypsin-kinase (0·5 mg. trypsin/10 cc. solution).*

Exp. No. / Fig. No.		1		2		3		4	
Total N (mg. in 10 cc. solution)		8·77		19·60		25·81		44·71	
Total P (mg. in 10 cc. solution)		0·460		0·937		1·350		2·250	
(Time mins.)		N	P	N	P	N	P	N	P
10	15*	4·66	0·334	3·08	0·384	3·43	0·203	4·41	0·25
20	30	6·50	0·421	6·37	0·609	5·74	0·397	8·02	0·48
30	45	7·00	0·460	8·99	0·745	8·16	0·570	10·33	0·66
40	60	7·66	0·461	10·82	0·857	10·37	0·740	13·30	0·99
50	75	8·18	0·463	12·49	0·916	12·01	0·850	16·69	1·20
60	90	8·60	0·463	14·06	0·930	13·62	0·991	18·45	1·36
70	105	8·54	—	14·81	—	14·14	1·00	21·60	1·60
80	120	—	—	15·29	—	14·70	1·01	21·81	1·66
90	135	—	—	15·65	—	15·48	1·04	24·50	1·73
100	150	—	—	16·35	—	16·28	1·11	26·08	1·84
110	165	—	—	16·45	—	17·85	1·14	20·83	1·87
120	180	—	—	—	—	17·92	—	27·55	1·93

* 15 minute intervals refer only to Exp. 4.
N = mg. acid-soluble N per 10 cc. solution.
P = mg. acid-soluble P per 10 cc. solution.

are shown in Table I A and curves showing the changes graphed as percentages of the total nitrogen and phosphorus are given in Figs. 1–4. The N/P ratios of the trichloroacetic acid precipitate at successive intervals of time were obtained by difference and are shown in Table II.

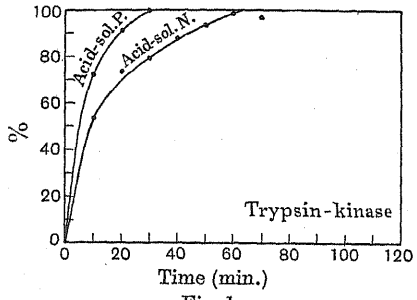


Fig. 1.

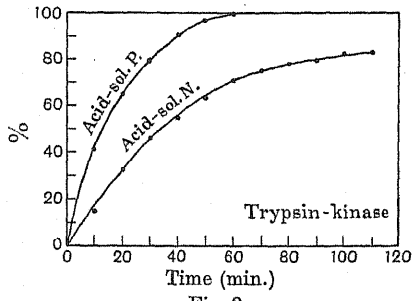


Fig. 2.

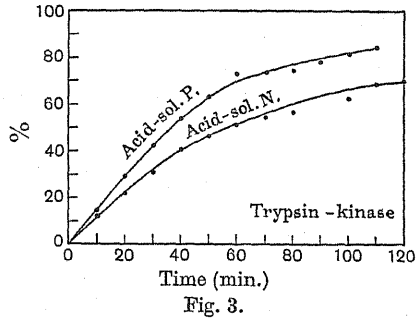


Fig. 3.

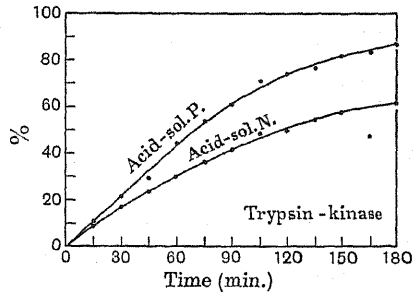


Fig. 4.

Hydrolysis by pepsin.

Caseinogen solution. This was made up in a manner similar to that for trypsin-kinase except that dilute HCl was used instead of alkali.

Enzyme solution. This was a 1 % filtered solution of granular pepsin (B.D.H.) the appropriate quantity of enzyme being obtained by dilution.

Table I B. *Pepsin (15 mg./10 cc. solution).*

Exp. No. / Fig. No.	5		6		7		8	
Total N (mg. in 10 cc. solution)	9·66		12·36		18·34		22·72	
Total P (mg. in 10 cc. solution)	0·515		0·652		0·959		1·23	
(Time mins.)	N	P	N	P	N	P	N	P
10	4·38	0·095	5·14	0·082	4·52	0·071	4·34	0·071
20	5·67	0·155	6·72	0·168	6·51	0·129	6·62	0·095
30	6·20	0·177	7·56	0·215	7·95	0·186	7·14	0·176
40	6·23	0·194	7·81	0·239	8·82	0·233	9·10	0·231
50	6·27	0·192	8·16	0·257	9·27	0·254	9·00	0·255
60	6·37	—	7·91	0·262	9·62	0·275	9·87	0·274
70	—	—	—	—	10·08	0·284	10·50	0·301
80	—	—	—	—	10·85	0·288	—	—
90	—	—	—	—	10·92	0·298	—	—
100	—	—	—	—	10·85	0·306	—	—
110	—	—	—	—	10·96	0·312	—	—

N = mg. acid-soluble N per 10 cc. solution.
P = mg. acid-soluble P per 10 cc. solution.

Buffer solution. The KCl-HCl mixture (p_H 1·8) of Clark and Lubs was used. The p_H optimum for pepsin as given by Northrop [1922] is 1·5–2·5 and our solutions throughout the course of the digestions were within this range.

Procedure: as for trypsin-kinase with the addition of one experiment in which six tubes containing the same amount of caseinogen but varying amounts of enzyme were incubated for 90 minutes. At the end of that time the amounts of acid-soluble nitrogen and phosphorus were estimated. This constituted Exp. 9.

Results. These are shown in Tables I B and II and in Figs. 5–8. The results of Exp. 9 are given in Table III.

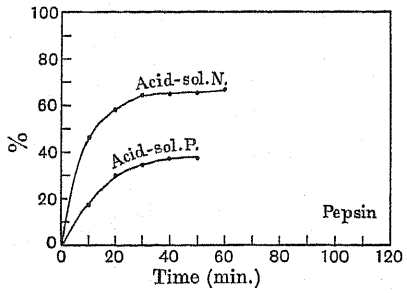


Fig. 5.

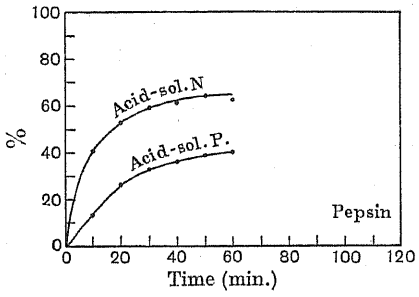


Fig. 6.

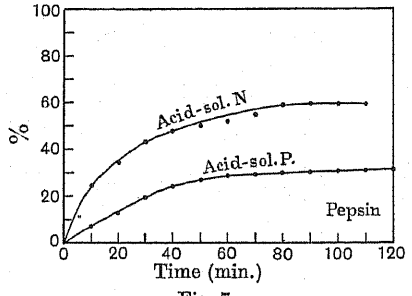


Fig. 7.

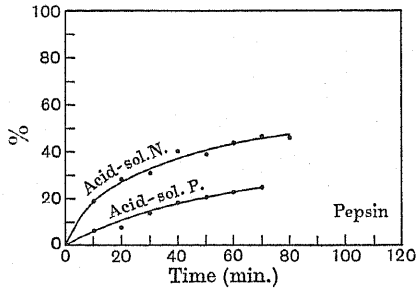


Fig. 8.

Table II. *N/P ratios of trichloroacetic acid precipitates.*

Exp. No.		Trypsin-kinase				Pepsin			
Time (mins.)		1	2	3	4	5	6	7	8
10	15*	32·6	29·9	19·5	20·2	12·6	13·1	15·6	15·8
20	30	58·2	40·3	21·1	20·8	11·1	12·2	14·3	14·1
30	45	∞	55·3	22·6	21·7	10·3	11·6	13·4	13·7
40	60	—	109·7	25·3	24·9	10·7	11·7	13·1	13·6
50	75	—	338·6	27·6	26·7	10·5	11·4	12·9	14·0
60	90	—	791·4	34·0	29·6	—	12·1	12·7	13·4
70	105	—	—	33·3	35·6	—	—	12·2	13·2
80	120	—	—	32·7	38·8	—	—	11·2	—
90	135	—	—	—	39·6	—	—	11·2	—
100	150	—	—	35·5	46·1	—	—	11·3	—
110	165	—	—	37·9	—	—	—	11·3	—
120	180	—	—	—	54·5	—	—	—	—

* 15 minute intervals refer only to Exp. No. 4.

Table III. *Exp. 9.*

Total N (10 cc. solution) 15·40 mg. Total P (10 cc. solution) 0·770 mg.
Each tube incubated for 90 minutes.

Pepsin (mg. per 10 cc. solution)	Acid-soluble N (mg. per 10 cc. solution)	Acid-soluble N Percentage total N	Acid-soluble P (mg. per 10 cc. solution)	Acid-soluble P Percentage total P	N/P ratio of trichloroacetic acid ppt.
5	6·70	43·5	0·107	14·0	13·12
10	7·96	51·7	0·184	24·0	12·70
15	8·85	57·5	0·248	32·0	12·54
20	8·94	58·1	0·276	36·0	13·08
25	9·47	61·5	0·306	40·0	12·77
30	9·60	62·3	0·315	41·0	12·75

Discussion.

The graphs show the relative rates of liberation of acid-soluble nitrogen and phosphorus during the digestion of caseinogen. In no case, either with trypsin-kinase or with pepsin, is it found that acid-soluble nitrogen and phosphorus are produced at the same rate, and it is therefore apparent that the first step in the degradation of caseinogen by proteolytic enzymes does not result in products which are completely soluble in trichloroacetic acid. This is quite contrary to the assumption made by Northrop [1924]. The fact that Northrop's assumption is unjustified is shown more clearly by Table II, in which are summarised the N/P ratios of trichloroacetic acid precipitates at successive intervals of time. It is quite apparent from these ratios that solubility in trichloroacetic acid is no index of the first stage of the degradation of caseinogen either by trypsin-kinase or pepsin. The caseinogen molecule can undergo very considerable disruption before becoming completely soluble in trichloroacetic acid and Northrop's work on the kinetics of trypsin action will have to be reconsidered from this new point of view.

Haldane [1930], commenting on Northrop's work, draws attention to his finding that the effect of substrate concentration is quite different according as the total action is measured by change in conductivity or production of amino-nitrogen, or by the actual destruction of caseinogen. Haldane suggests that this result may be due to the fact that Northrop's trypsin solutions contained carboxypolypeptidase and aminopolypeptidase (erepsin). These enzymes do not attack molecules of magnitude greater than polypeptides and would therefore play no part in the change from acid-insoluble to acid-soluble. A second alternative is suggested by Haldane, namely that trypsin has a greater affinity for the degradation products of caseinogen than for the protein itself. Such a hypothesis would be necessary to explain Northrop's finding that the amount of substance rendered soluble in trichloroacetic acid was almost directly proportional to the concentration of caseinogen between 1·5 and 5 % if the substance precipitated by trichloroacetic acid were wholly caseinogen. In our experiments this was certainly not the case and moreover we have found that the rate of liberation of acid-soluble nitrogen falls off

slightly with increase in caseinogen concentration. From the point of view of enzyme kinetics it is necessary that Northrop's work should be repeated using a method which will separate caseinogen completely from its cleavage products.

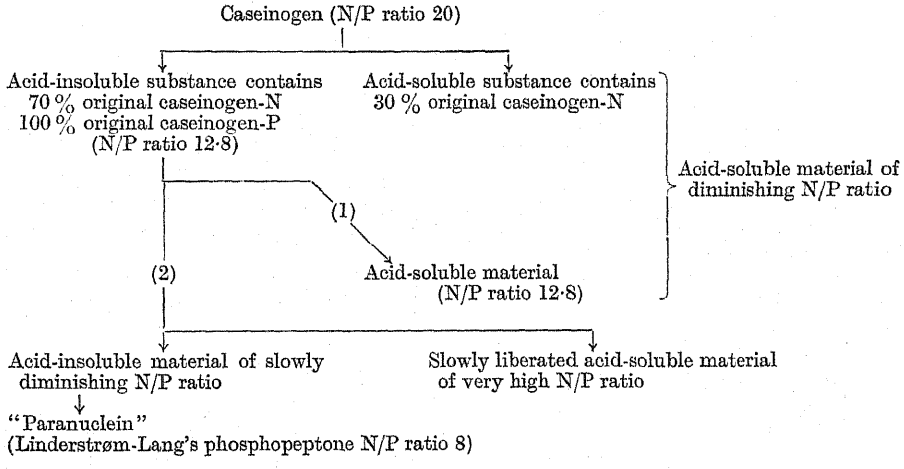
It will be seen from Figs. 1 to 8 that with trypsin-kinase acid-soluble nitrogen is liberated more slowly than acid-soluble phosphorus while, with pepsin, the reverse is true. This appears to indicate that the action of the two enzymes is fundamentally different, evidence for which can best be obtained by treating the data for each enzyme separately.

Trichloroacetic acid appears to separate peptic digests of caseinogen, in the initial stages of the hydrolysis of the protein, into two fractions: (1) insoluble in trichloroacetic acid and relatively rich in phosphorus (N/P ratio less than that of caseinogen), (2) soluble in trichloroacetic acid and relatively poor in phosphorus (N/P ratio greater than that of caseinogen). It is known that, at any one instant, several reactions are proceeding side by side during proteolysis and it might be expected that both the acid-soluble and acid-insoluble fractions would consist of mixtures of substances at various stages of degradation. That this is not the case, so far as the acid-insoluble material is concerned, may be ascertained from Table III. Here it is seen that, although the acid-soluble phosphorus has increased from 14 to 41 % of the total and the acid-soluble nitrogen from 44 to 62 %, the N/P ratio of the acid-insoluble material has remained practically constant. It is to be concluded therefore that, from the stage represented by tube No. 1 to that represented by tube No. 6 only one acid-insoluble substance was present and that the increase in acid-soluble material between these two stages resulted from the splitting of the acid-insoluble substance into two or more substances completely soluble in trichloroacetic acid.

Thus at least 86 % of the phosphorus of caseinogen can be split off in the form of a body insoluble in trichloroacetic acid and having N/P ratio of 12·8. From the fact that the precipitate from tube No. 1 had a N/P ratio of 13·1 we may infer that very little, if any, caseinogen still remained unattacked by the enzyme, and it is to be presumed that, in the 90 minutes which had elapsed since the beginning of the digestion, part of the non-caseinogen acid-insoluble material had acted as substrate and had contributed to the formation of acid-soluble phosphorus. It seems reasonable to conclude, therefore, that the initial action of pepsin on caseinogen is to split the protein into substances one of which is insoluble in trichloroacetic acid and contains all the phosphorus, while the other is soluble in trichloroacetic acid and contains no phosphorus. Although Exp. 9 demonstrates the cleavage of this phosphorus-containing acid-insoluble substance (N/P ratio 12·8) into fractions which are completely soluble in trichloroacetic acid it appears that this is not the only mode of degradation. This is shown by our data on the time-course of the N/P ratio of the trichloroacetic acid precipitate with varying concentrations of caseinogen as summarised in Table II. It will be seen that, in Exps. 5 and 6, the only acid-insoluble material present after 10 minutes' digestion is the

substance of N/P ratio 12·8 already mentioned. In Exps. 7 and 8 some substance of higher N/P ratio (probably unchanged caseinogen) is still present even after 10 minutes. Every experiment, however, shows a slow change in N/P ratio with time, a fact which indicates that here we have to deal with the cleavage of the acid-insoluble material (N/P ratio 12·8) into two fractions, (1) insoluble in trichloroacetic acid and low N/P ratio, (2) soluble in trichloroacetic acid and high N/P ratio. On the basis of viscosity measurements Linderstrøm-Lang [Holter *et al.*, 1932] has suggested that the mode of attack of the enzyme on its substrate may be different at different stages of digestion and our work would appear to confirm this. It may also be noted that a similar slow breakdown is shown in Table XII of Linderstrøm-Lang's paper. The phosphopeptone (paranuclein) investigated by Linderstrøm-Lang had a N/P ratio 8 and would appear to be a later stage of digestion than our acid-insoluble material of ratio 12·8. It was noted from Table II that the N/P ratio of the acid-insoluble material was tending towards the value obtained by Linderstrøm-Lang and it is possible that paranuclein is derived from our acid-insoluble substance by the second mode of breakdown suggested. It has to be remembered that Linderstrøm-Lang's material was characterised by being insoluble at acid reaction in the absence of trichloroacetic acid and that in his experiments the p_H of the system was not kept constant throughout the digestion. This may explain the fact that his phosphopeptone accounted for about 70 % of the total protein-phosphorus while in our Exp. 9 less than 60 % of the phosphorus is acid-insoluble and it is still in the form of the substance with a N/P ratio 12·8. The mode of breakdown seems therefore to depend on the composition of the system.

On the basis of these findings a provisional scheme for the hydrolysis of caseinogen by pepsin might be suggested as below:



(1) and (2) represent simultaneous modes of breakdown which may proceed at different relative rates according to conditions.

The course of the digestion of caseinogen with trypsin-kinase seems to be altogether different from that with pepsin. It will be seen from Figs. 1 to 4 that, with the former, the rate of liberation of acid-soluble phosphorus is greater than that of acid-soluble nitrogen. The action of the enzyme on caseinogen and on its immediate degradation products is characterised by the production of two fractions (1) insoluble in trichloroacetic acid and poor in phosphorus, (2) soluble in trichloroacetic acid and rich in phosphorus. As will be seen from Table II the N/P ratio of the trichloroacetic acid precipitate is increasing rapidly in the case of trypsin-kinase while it is decreasing slowly with pepsin. Since solubility in trichloroacetic acid may be taken as a rough guide to molecular size, it would appear that with trypsin-kinase phosphorus is liberated in comparatively small molecules but, with pepsin, in the form of large molecules. The differences in behaviour of the two enzymes can best be explained by the assumption that all the phosphorus of caseinogen is concentrated in one relatively small group. The phosphorus linkages would therefore appear to direct to some extent the action of proteolytic enzymes—trypsin-kinase effecting rupture of peptide linkages in proximity to phosphorus atoms and so confining the phosphorus to the cleavage product of lower molecular weight; the action of pepsin being relegated to peptide linkages distant from phosphorus atoms and giving rise to phosphorus-containing substances of high molecular weight. That groups within the molecule can exert a controlling influence over the action of enzymes is shown by the necessity for a free carboxyl or a free amino-group for the action of the polypeptidases [Grassmann and Dyckerhoff, 1928].

The further course of the hydrolysis with trypsin may be ascertained from Exps. 1 and 2 in which it was found possible to obtain all the phosphorus in an acid-soluble condition while about 20 % of the nitrogen remained insoluble. It is possible that the first action of trypsin-kinase on caseinogen results in the formation of two fractions, one acid-soluble and containing all the phosphorus, the other acid-insoluble and containing no phosphorus, the acid-insoluble phosphorus of the successive intervals of Exps. 1 to 4 being ascribed to unchanged caseinogen. The phosphorus-containing fraction would approximate to the size of Rimington's phosphopeptone which, it will be remembered, was isolated in amount representing over 50 % of the original caseinogen phosphorus. Rimington's compound is more likely to be a primary cleavage product of the caseinogen molecule than a secondary product resulting from the hydrolysis of a paranuclein as suggested by Linderstrøm-Lang [Holter *et al.*, 1932].

It is difficult to see how our results can be interpreted otherwise than as indicating that all the phosphorus is concentrated in one relatively small group in caseinogen, whether the latter is a single molecule or an association of molecules. If, as Linderstrøm-Lang suggests, caseinogen is an association of various proteins containing different amounts of phosphorus, it is impossible to account for the formation of one acid-insoluble substance containing over

86 % of the total phosphorus during peptic digestion. The possibility must therefore be seriously entertained that Linderstrøm-Lang's alcoholic-HCl has a chemical effect on caseinogen, and that his various fractions are not homogeneous.

SUMMARY.

The action of trypsin-kinase and pepsin on the caseinogen molecule results in the formation of products which are not completely soluble in trichloroacetic acid.

With pepsin the rate of liberation of acid-soluble nitrogen is greater than that of acid-soluble phosphorus while with trypsin-kinase the opposite is true.

These results are explained on the basis of the existence of a caseinogen molecule with all the phosphorus concentrated in one comparatively small group.

The action of trypsin-kinase is considered to be on a peptide linkage in proximity to the phosphorus atoms thus confining all the phosphorus to the cleavage product of lower molecular weight. The action of pepsin is on a peptide linkage distant from the phosphorus atoms giving rise to a phosphorus-containing substance of high molecular weight.

The formation of Rimington's phosphopeptone is considered to be specific for the action of trypsin-kinase and that of paranuclein for the action of pepsin.

We have to thank Dr N. C. Wright for his interest in this work and for supplying us with caseinogen.

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CCXXXVII. NOTE ON THE OXIDATION OF TYROSINE, TYRAMINE AND PHENYLALANINE WITH HYDROGEN PEROXIDE.

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Guggenheim [1913] has shown that the pods of the broad bean (*Vicia faba*) contain 3:4-dihydroxyphenylalanine, and Miller [1920] has isolated it from the Georgia speckled velvet bean (*Stizolobium deeringianum* Bort). It has also been found in insects [Schmalfuss and Müller, 1927; Przibram, 1922; Schmalfuss, 1927]. Recently, Schmalfuss and Heider [1931] isolated 3:4-dihydroxyphenylethylamine from the pods of the common broom (*Sarothamnus scoparius* Wimm) and found also that it was accompanied by tyramine.

The mode of origin in the plant or animal of these catechol derivatives is unknown, but it does not seem improbable that 3:4-dihydroxyphenylalanine is produced by the oxidation of tyrosine and 3:4-dihydroxyphenylethylamine by the oxidation of tyramine. It might be thought that tyrosinase would be the natural agent to bring about these oxidations, since it has been shown to produce *ortho*-dihydric from monohydric phenols [Pugh and Raper, 1927] and 3:4-dihydroxyphenylalanine has been isolated from the products of its action on tyrosine [Raper, 1926]. On the other hand, tyrosinase acts so rapidly on catechol derivatives that when produced by its action they would not tend to accumulate but be quickly oxidised further. This difficulty concerning their origin in living organisms could be overcome if tyrosinase consisted of two separate enzymes, one of which oxidised monohydric to *ortho*-dihydric phenols and the other oxidised only the *ortho*-dihydric phenols. If the first enzyme could act under conditions unsuitable for the second, then the catechol derivative under these conditions might accumulate. Pugh [1930] has shown, however, that there is no evidence that such a monophenol oxidase exists as a separate component of tyrosinase, and as this is the only enzyme known at present which oxidises monophenolic substances to catechol derivatives it is probable that some other agent is responsible for the oxidation of tyrosine and tyramine to their corresponding 3:4-dihydroxy-compounds.

In the course of experiments on the oxidation of *l*-tyrosine in dilute solution by hydrogen peroxide in the presence of various metallic catalysts it was observed that a trace of ferrous sulphate caused a fairly rapid oxidation of the amino-acid. Examination of the brown solution resulting from the

oxidation showed that it contained a substance giving a marked green colour with ferric chloride changing to reddish purple on the addition of sodium acetate. The substance responsible for this reaction was isolated and found to be *l*-3:4-dihydroxyphenylalanine, identical with the naturally occurring amino-acid. Under similar conditions it has also been found that tyramine is readily oxidised and yields 3:4-dihydroxyphenylethylamine, which was isolated as its tribenzoate.

Dakin and Herter [1907], studying the oxidation of phenylalanine, have shown that the use of hydrogen peroxide under more drastic conditions than were employed in the present experiments causes oxidation in both side-chain and nucleus. It seemed of interest to determine whether under milder conditions of oxidation the side-chain could be kept intact and the aromatic nucleus alone oxidised as with tyrosine and tyramine. To a small extent this has been found possible. By oxidising phenylalanine under the same mild conditions which were used with tyrosine and tyramine a small amount of tyrosine was obtained. This is of interest, since in alcaptonuria homogentisic acid has been shown to be produced from phenylalanine as well as from tyrosine, and Medes [1932] has recently described a condition in which phenylalanine is converted into *p*-hydroxyphenylpyruvic acid *via* tyrosine.

Several attempts have been made by the author to obtain homogentisic acid by the oxidation of *p*-hydroxyphenylpyruvic acid by hydrogen peroxide and iron, but they have all been unsuccessful.

These results show, therefore, that a possible mode of origin of *l*-3:4-dihydroxyphenylalanine and its corresponding base may be the oxidation of *l*-tyrosine and tyramine respectively by hydrogen peroxide under the catalytic influence of iron. They also demonstrate that tyrosine may be produced by the direct oxidation of phenylalanine and they add a few examples to the many which already exist of the probable importance of hydrogen peroxide as an oxidising agent in living organisms.

Experimental.

Oxidation of tyrosine.

To a solution of 2 g. tyrosine in 4 litres of water at room temperature were added 0·1 g. $FeSO_4, 7H_2O$ and 10 cc. (1·5 mols.) of 6 % H_2O_2. The solution soon became yellow and then gradually darkened to a brownish colour, depositing a little dark brown pigment. After standing for 3 days the solution was filtered and 10 g. lead acetate in 50 cc. water were added to the filtrate. This precipitates most of the brown pigment. The precipitate was filtered off and rejected. The filtrate on making definitely alkaline with ammonia gave a bulky precipitate. This was filtered off, washed a few times with cold water and then, after suspending in 200 cc. water, decomposed with H_2S. After removing the lead sulphide the solution was concentrated by distillation under reduced pressure until a good crystalline deposit had separated. The solution

was then heated to dissolve the deposit, transferred to a beaker, allowed to cool, neutralised to Congo red and concentrated to about 15 cc. in a vacuum desiccator over sulphuric acid. 0·32 g. of crystalline 3:4-dihydroxyphenylalanine was thus obtained; M.P. 275–80° (decomp.). By evaporating the mother-liquor to very small bulk a further crop of crystals of the amino-acid separated. In all 0·58 g. of crude dihydroxyphenylalanine was thus obtained. One recrystallisation from boiling water containing a trace of acetic and sulphurous acids gave a product with M.P. 279–80° (decomp.). This is the M.P. of the laevo-form of the amino-acid. It was greenish in colour due to traces of iron which are not completely removed in the process of separation *via* the lead salt. Attempts to remove the iron by decomposing the lead salt in presence of enough ammonia to render the solution alkaline resulted in a colourless product but with a lower M.P. 269–70°, this being due to racemisation. A sample of the racemised amino-acid obtained in this way, M.P. 268–9° (decomp.) was analysed: C, 55·2; H, 5·9; N, 7·1 %. $C_9H_{11}O_4N$ requires C, 54·8; H, 5·6; N, 7·1 %. By recrystallising the product of M.P. 279–80° three times from weakly acidulated water as above, crystals were obtained with only a slight colour. These were examined polarimetrically. 0·15 g. in 3·05 cc. *N* HCl gave $[\alpha]_{5461}^{18°} = -13·6°$. This is the value found by Harington and Randall [1931] for pure *l*-3:4-dihydroxyphenylalanine.

The amino-acid reduced ammoniacal silver nitrate in the cold and gave an intense green colour with ferric chloride.

Oxidation of phenylalanine.

3 g. inactive phenylalanine were dissolved in 3 litres of water and 0·15 g. $FeSO_4, 7H_2O$ added. 15 cc. of 6 % hydrogen peroxide were then introduced and the solution kept at room temperature until, after 4 days, the hydrogen peroxide had disappeared (chromic acid test). The solution had become deep brown and there was a brownish-black deposit. It was filtered, concentrated by distillation under reduced pressure to about 500 cc. and then 80 cc. of 20 % lead acetate were added together with 2*N* ammonia until no further precipitate came down. The lead precipitate was filtered off, washed and decomposed with H_2S in 600 cc. water. The PbS was removed by filtration, well washed and the filtrate and washings concentrated by distillation under reduced pressure to about 50 cc. and then, after neutralising to Congo red with ammonia, in a vacuum desiccator. A crystalline deposit was obtained which was recrystallised from boiling water, clarifying with charcoal. The substance crystallised in fine needles exactly like tyrosine; M.P. 287° (decomp.). Analysis, C, 59·3; H, 5·5; N, 7·6 %. $C_9H_{11}O_3N$ requires C, 59·7; H, 6·1; N, 7·7 %. The product was readily oxidised by tyrosinase from mealworms giving the typical colour changes of tyrosine. It also gave Millon's reaction. *o*- and *m*-Tyrosine both have a lower M.P. than that of the oxidation product and are not oxidised by tyrosinase from mealworms [Abderhalden, 1928]. From 4 g. of phenylalanine 167 mg. of the crude and only 43 mg. of the recrystallised tyrosine, M.P. 287°,

were obtained. The mother-liquor from which the first crop of tyrosine was obtained gave a further crop of crystals on concentration consisting largely of tyrosine as judged by the M.P. (270° after recrystallisation). The final mother-liquor gave a reddish-violet colour with ferric chloride suggesting the presence of *o*-tyrosine, but this was not confirmed in a second experiment. It is doubtful therefore whether *o*-tyrosine is produced along with the relatively more abundant *p*-tyrosine.

Oxidation of tyramine.

To 2 g. tyramine hydrochloride in 2 litres of water 0·05 g. $FeSO_4,7H_2O$ was added and then 60 cc. of 1 % H_2O_2 (1·5 mol.). The solution was allowed to stand overnight at room temperature, after which the H_2O_2 had disappeared. It was brownish-coloured and gave a marked catechol reaction with ferric chloride. 10 cc. of sulphurous acid were added and the solution was concentrated by vacuum distillation to about 100 cc. 50 cc. of 20 % lead acetate were added and then 2*N* ammonia until no further precipitate was formed. The lead precipitate was filtered off by suction on a pulp filter, washed with a little water and the paper pulp and precipitate suspended in 400 cc. water. The lead compound was now decomposed with H_2S, the lead sulphide and pulp removed by filtration and washed with hot water. The filtrate and washings were concentrated by vacuum distillation to about 30 cc. and then, after addition of 1·5 cc. concentrated HCl, in a vacuum desiccator. The dark brown crystalline residue was extracted with 20 cc. hot alcoholic HCl, filtered and the filtrate concentrated in a vacuum desiccator. A small amount of a sticky brown substance separated and was filtered off. The filtrate was now taken nearly to dryness in the desiccator when a brownish crystalline mass separated. This was filtered off, washed with a little absolute alcohol and dried *in vacuo*; 0·45 g. was obtained. It gave a strong catechol reaction with ferric chloride and was presumably the crude hydrochloride of 3:4-dihydroxyphenylethylamine. It was dissolved in a few cc. of water and benzoylated, using 5 g. benzoyl chloride and 30 cc. 10 % NaOH. The benzoylated base which separated was shaken out with benzene. On distilling off the benzene a viscous residue remained. This was redissolved in 5 cc. benzene and allowed to stand overnight in order to remove any tyramine dibenzoate, which is only slightly soluble in benzene. None was obtained. The solution was now allowed to evaporate slowly until crystals appeared. The crystallisation was aided by the cautious addition of light petroleum. The crystals thus obtained were filtered off, washed with a mixture of 3 parts benzene and 1 part light petroleum and dried *in vacuo*; M.P. 136–7°. On recrystallisation twice from 80 % aqueous alcohol and again from benzene and light petroleum a product was obtained with M.P. 140–1°. This is the M.P. of the tribenzoate of 3:4-dihydroxyphenylethylamine [Schmallfuss and Heider, 1931]. Micro-analysis, C, 74·6; H, 5·15; N, 3·2 %; $C_{29}H_{23}O_5N$ requires C, 74·8; H, 4·9; N, 3·0 %.

SUMMARY.

By the action of hydrogen peroxide with ferrous sulphate as a catalyst it is shown that phenylalanine yields tyrosine, *l*-tyrosine yields *l*-3:4-dihydroxyphenylalanine and tyramine yields 3:4-dihydroxyphenylethylamine.

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CCXXXVIII. THE METABOLIC ACTIVITY OF THE CELLS OF THE TRIGEMINAL GANGLION.

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(*Received October 26th, 1932.*)

We have so far but little information about the relative metabolic powers of the different parts of the central nervous system. Kinnersley and Peters [1930] have observed that the concentration of lactic acid differs in different parts of the brain of the avitaminous pigeon, though not in the different parts of that of the normal bird. Gavrilescu and Peters [1931] have found that different parts of the pigeon's brain take up oxygen at different rates. Some information is available [Holmes, 1930] about the metabolic activity of the different parts of the neurone. The white fibres in the central nervous system, and those which form the peripheral nerves, appear to differ very materially in their oxidative powers from what we may, for the moment, call the "cell complex," that is, the cell body, the dendrites, and the junctional or synaptic tissue. It was in the hope of analysing a little further the activity of this "cell complex" that the work here described was undertaken. In the brain and spinal cord itself, the cell is so closely associated with dendrites and, presumably, with synapses, that no mechanical separation is possible. In the posterior root ganglia, however, cells and fibres are found, but the dendrites and axons, which are both to all appearances ordinary medullated fibres, pass out of the ganglia without making contact with other neurones. These ganglia, therefore, afford material for the examination of the activity of the cell bodies, uncomplicated by that of non-medullated dendrites or synaptic tissue. On the other hand, to make use of this tissue for the study of the metabolism of nerve cells, and to draw deductions from the results as to the behaviour of nerve cells in general, involves the assumption that the cells are similar in metabolic behaviour to others in other parts of the central nervous system; and this assumption is not necessarily justified, for the cells are larger than, and differ in form from, most of the cells in the central nervous system. However, it must be remembered that the latter present an enormous variety of shapes and sizes, ranging from the large pyramidal Betz cells of the motor cortex and the rounded Purkinje cells of the cerebellum to the small pyramidal cells which are present in such large numbers in the grey matter as a whole.

The experiments present other difficulties. The most convenient tissue to use was found to be the trigeminal ganglia of sheep and oxen. These bodies

contain, besides nerve cells, considerable amounts of white fibres, and of connective tissue. It is difficult to assess accurately the exact proportion of cellular substance present. A glance at a fixed and stained section, however, shows that the cells occur in large masses, and that they are closely packed, and it seems unlikely that their metabolic activity could be seriously masked by the presence of the relatively inert white fibres and connective tissue. In the light of what follows, it is noteworthy that a brief inspection of a section of cerebral cortex stained, for instance, with methylene blue shows that even here the cell density is not very great. The cells are much smaller than those of the posterior root ganglia and are dotted about amid a mass, not in this case of white fibres, but of non-medullated processes. If, therefore, the trigeminal ganglion cells may be taken as typical nerve cells, we have a fair chance of examining their biochemical behaviour.

The experimental procedure was as follows. Sheep's heads (usually three), were obtained fresh from the slaughter house. The skulls were sawn open, and the brains removed, the Vth cranial nerve being cut through as near as possible to the brain. The trigeminal ganglia, which lie on either side of the cavernous sinus, surrounded by tough dura mater and bone, were exposed and removed by the use of saw, bone forceps, and scissors. The three divisions of the trigeminal nerve were cut through close to the ganglion. The six ganglia were chopped with scissors, and sampled. Portions of the cranial nerves, of white matter and of cerebral cortex were usually taken for comparison. The oxygen uptakes were measured in the usual way, in the Barcroft apparatus. The tissue was suspended in Ringer's solution buffered with 1/5 volume of $M/2$ sodium dihydrogen phosphate adjusted to p_H 7·6. On every occasion, the Barcroft cups were filled with oxygen. Whenever possible, 0·5 g. of tissue was placed in each apparatus.

The curves obtained were, on the whole, far less satisfactory than is usual when dealing with other parts of the central nervous system. This was due, probably (1) to the very low rate of oxygen consumption; (2) to the difficulty of sampling. The connective tissue, which binds together the elements of the ganglion is extremely tough. It is, in consequence, difficult to chop the organ satisfactorily, and the tissue tends to collect in lumps in the Barcroft cups. In spite of these difficulties, it is at once plain that the oxygen consumption of ganglion tissue is of a very low order, much the same, in fact, as that of the peripheral nerve of the same animal. Samples of cortex taken at the same time as the ganglion, showed the usual large uptake, and there is no reason to suppose that the ganglion cells had suffered more manipulative damage than did those of the cortex. Fig. 1 shows the oxygen uptakes of trigeminal ganglion, postganglionic cranial nerve (V), and cortex—sheep's tissue being used in each case.

In only one experiment was a much larger oxygen uptake than this recorded, this being a single experiment in which ox tissue was used, when the uptake after 150 minutes amounted to 421 mm.^{3} This was quite an excep-

tional observation, and even so, the oxygen consumed amounted to only about a quarter of that consumed by sheep's cortex.

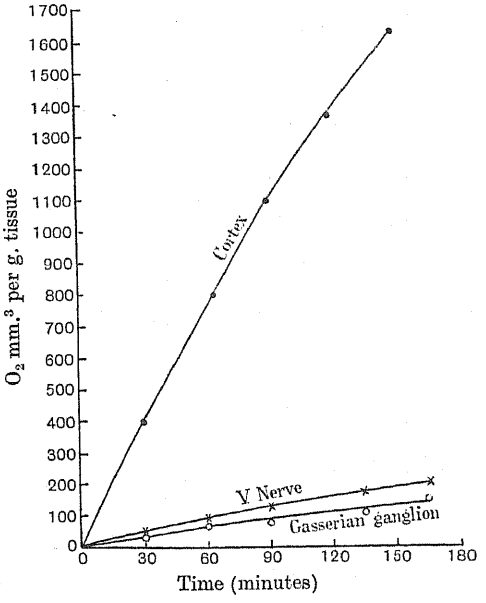


Fig. 1.

Table I.

Date	Tissue		Uptake after 150 mins. (mm.^{3}/g.)
1. iii. 32	Trigeminal ganglion (sheep)		132
8. iii. 32	,,		140
,,	,,		98
15. iii. 32	,,		170
,,	,,		128
22. iii. 32	,,		236
29. iii. 32	,,		178
		Average	154
8. iii. 32	Nerve (sheep)		86
15. iii. 32	,,		118
,,	,,		128
29. iii. 32	,,		114
		Average	112

Table I shows figures observed for total oxygen consumption both of ganglia and of cranial nerves. The values recorded for the uptake of the ganglia varied so much from experiment to experiment, even when the tissue was taken from the same mass of chopped ganglion, that it is impossible to say with certainty whether the oxygen consumption is or is not increased by the addition of lactate. That no large increase (such as may be observed with cortex) takes place is certain, but the experiments throw no light on the question as to whether or not a slight increase occurs. With succinate, on the

other hand, there is a very large increase. For instance, after 150 minutes, the oxygen uptake of sheep's trigeminal ganglion in Ringer and succinate ($M/20$) was 790 mm.^{3} per g. Quastel and Wheatley [1932] have shown that both grey and white matter readily change succinate to *l*-malate. The increase was therefore not necessarily due to the activity of the cells.

Since the oxidative powers of the nerve cells in question are evidently not of a high order, it was surprising to find that they readily gave a strong "Nadi" reaction. This is easily observed by freezing and cutting sections of the fresh ganglia, placing them in the "Nadi" reagent for a short time and then examining them under the low power of the microscope. The cells will be seen to be stained a deep blue colour, while the other tissue elements are nearly colourless. They, too, eventually become blue, but the colour is less intense than in the case of the cells and develops much more slowly. Incidentally, it was noticed that cooling to temperatures obtained with an ordinary acetone spray very much reduced the power of cortical tissue to consume oxygen.

The glycolytic power of the ganglion cells was also examined. A sample (0·7 g.) of tissue was placed in each of the two cups of a large Barcroft apparatus. One cup contained plain Ringer, the other Ringer containing 0·5 % glucose. The apparatus was filled with oxygen-free nitrogen and shaken for 3 hours in a thermostat at 37°. For purposes of comparison, samples of white matter and of cerebral cortex were treated in exactly the same way. The tissue, at the end of the experiment, was treated with trichloroacetic acid, made up to volume, filtered, and lactic acid estimated as usual. (Calcium hydroxide-copper sulphate precipitation: lactic acid estimated according to Friedemann, Cotonio and Shaffer [1927]).

The results are given in Table II. It will be seen that, in every case, less lactic acid is formed by the ganglia even than by white matter; cortex shows its usual (and marked) glycolytic power.

Table II. *Mg. of lactic acid formed per g. of tissue, after shaking for 3 hours anaerobically, with and without glucose.*

Ringer buffered with phosphate p_H 7·6.

Ganglion			White matter			Cortex		
Glucose absent	Glucose present	Formed from glucose	Glucose absent	Glucose present	Formed from glucose	Glucose absent	Glucose present	Formed from glucose
1·60	2·21	0·61	1·30	3·15	1·85	1·46	8·64	7·17
1·02	2·04	1·02	0·66	2·89	2·23	1·00	13·4	12·40
1·47	3·58	2·11	1·20	4·42	3·22	1·02	10·44	9·42
		1·24			2·43			9·66

These results show that mammalian sensory ganglia have feeble powers of oxidation and of glycolysis, when compared with the cerebral cortex. It has already been pointed out that the experiments on oxygen consumption are not without technical difficulties. Nevertheless, I venture tentatively to put

forward the hypothesis that, so far at least as oxidation and glycolysis are concerned, the chief seat of metabolic activity in the neurone is neither the medullated axon nor the cell body, but the dendritic and synaptic structures (supposing the latter to have a real and separate existence) which form such a large proportion of the bulk of the grey matter of the central nervous system.

It is of interest that Bethe [quoted by Evans, 1930], has found that the reflex mechanism of the tentacle of *Carcinus menas* continues to function, even after all the cell bodies have been cut away from the ganglion upon which the reflex depends, while Steinach [see Evans, 1930] has found that the posterior root ganglia of the rabbit continued to conduct impulses even after all the cells were dead and degenerated as a result of the cutting off of their blood supply. These findings, as Evans points out, suggest that the cell body is not essential for the conduction of impulses through the neurone, provided, of course, as in these cases, its destruction does not involve any actual interruption of continuity. But if the function of the neurone, as a unit, depends on its metabolic processes, these (or at any rate those of them which are important for function) must occur at some situation other than the cell body itself. We can eliminate, from previous studies, the medullated axon, and all that remains are the dendrites and synapses.

Summary.

The trigeminal ganglia of sheep show very poor powers of oxidation, although the cells contain indophenol oxidase. Their power of forming lactic acid from glucose is less than that of the white matter of the central nervous system. It is suggested that oxidation and glycolysis in brain occurs in the dendrites and synaptic structures, rather than in the cell bodies.

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CCXXXIX. OBSERVATIONS ON THE VARIATION OF p_H OF BRAIN TISSUE.

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In previous communications [Holmes and Holmes, 1927; Holmes and Sherif, 1932], it has been demonstrated that the amount of lactic acid formed by the excised brain after incubation is dependent, within limits, on the level of the blood-sugar at the time of death, while the lactic acid found in brains which have been rapidly fixed in liquid air is constant in amount and independent of the blood-sugar, except when the latter has been reduced by insulin to a low level. It was thought that it would be interesting to see whether these changes in lactic acid content were associated with any demonstrable change in the p_H of the tissue.

Mice were used for the experiments; the blood-sugar was raised, when desired, by the intraperitoneal injection of glucose, or lowered by means of subcutaneous injections of insulin. The animals were decapitated by a single stroke of a pair of strong, sharp scissors, and the heads were either allowed to fall into liquid air (which prevents further lactic acid formation, even after thawing), or were incubated for 1 hour (sometimes 45 minutes) at 37°. For p_H determinations the brains were removed from the skulls, placed in a small beaker or specimen tube, and rubbed to a fine suspension with 5·0 cc. of Ringer's solution. The p_H of the suspension was determined by means of the Cole potentiometer, gold electrodes and quinhydrone being employed. The standard acetate buffer was checked against a calomel cell. While the values obtained probably do not accurately represent the actual p_H of the tissue during life, there seems no reason to doubt that the differences observed were significant; and the results showed a very considerable constancy. It is curious that, in spite of the fact that the determinations were not made in an atmosphere containing a proportion of CO_2, yet the values observed were consistently lower (*i.e.* the tissue was more acid) than it could reasonably be expected to have been during life. The results are arranged in six groups, as follows.

(1) Animals with normal blood-sugar, decapitated and the heads frozen.

(2) Animals with normal blood-sugar, decapitated and the heads incubated.

(3) Animals decapitated after glucose injection, and the heads frozen.

(4) Animals decapitated after glucose injection, and the heads incubated.

(5) Animals decapitated during insulin hypoglycaemia, and the heads frozen.

(6) Animals decapitated during insulin hypoglycaemia, and the heads incubated.

It will be seen that the values observed in groups (1) and (3) are about the same. There is a fall (increase of acidity) on incubation in all cases, but in group (4) it is definitely greater than in group (2); in group (6) it is slightly less than in group (2). The values in group (5), where the brains were frozen during hypoglycaemia, are definitely higher (less acid) than in (1) (normals). In a certain number of cases in groups (5) and (6), estimations of the blood-sugar were performed, the blood from several animals, killed at the same time, being pooled for this purpose. It will be seen from the tables that, in general, the lower the blood-sugar, the higher is the p_H, both in brains frozen immediately and in those which have been incubated.

All the estimations of p_H were carried out at room temperature, which varied from 10 to 18°, in different experiments. The correction which would have to be applied, according to Adair, Cordero and Shen [1929], who were working with blood, to correct the values for this would be in such a direction as to make the values for 37° even more acid than those actually observed. In a few experiments which were performed at different temperatures, this

Table I.

Group (1). Normal animals. Decapitated, and heads dropped directly into liquid air.

Date	p_H observed
5. v. 32	6·868
,,	7·035
,,	6·868
12. v. 32	6·823
,,	6·858
,,	6·888
,,	6·892
,,	6·920
18. v. 32	6·923
Mean	**6·897**

Group (2). Normal animals. Decapitated, and heads incubated at 37° for 2 hours (Exp. of 5. v. 32) and 45 mins. (Exps. of 6. v. 32 and 19. v. 32).

Date	p_H observed
5. v. 32	6·754
,,	6·763
6. v. 32	6·665
19. v. 32	6·670
	6·677
Mean	**6·704**

Group (3). Animals given 0·5 cc. of 40 % glucose in water by intraperitoneal injection. Decapitated after 15 mins.; heads dropped directly into liquid air.

Date	p_H observed
30. v. 32	6·984
,,	6·984
,,	6·958
Mean	**6·975**

Group (4). Animals given 0·5 cc. of 40 % glucose in water by intraperitoneal injection. Decapitated after 15 mins.; heads incubated at 37° for 45 mins. (Exp. of 6. v. 32) and 60 mins. (Exp. of 30. v. 32).

Date	p_H observed
6. v. 32	6·510
,,	6·440
,,	6·391
30. v. 32	6·408
,,	6·454
,,	6·464
Mean	**6·444**

Table I (*contd.*).

Group (5). Animals starved for 24 hours. Insulin (1 unit) injected subcutaneously. Decapitated after various intervals of time. Heads dropped directly into liquid air.

Date	Interval before killing	Symptoms	p_H observed	Blood-sugar mg./100 cc.	Mean p_H for given blood-sugar
13. v. 32	2 hours	+	6·978	—	
,,	,,	+	7·042	—	
,,	,,	+	7·037	—	7·029
,,	,,	+	7·037	—	
,,	,,	+	7·055	—	
18. v. 32	15 mins.	−	6·976		
,,	,,	−	7·037	61·2	6·955
,,	,,	−	6·853		
,,	45 mins.	−	6·966		
,,	,,	−	7·028	46·3	7·005
,,	,,	−	7·023		
,,	1 hr. 55 mins.	+	7·063		
,,	,,	+	7·073		
,,	,,	+	7·041	48·5	7·077
,,	,,	+	7·019		
,,	,,	+	7·079		
,,	,,	+	7·188		

Group (6). Mice starved for 24 hours. Given insulin as indicated in table. Decapitated, and heads incubated 1 hour at 37°.

Date	Insulin units	Interval before killing	Symptoms	p_H observed	Blood-sugar mg./100 cc.	Mean p_H for given blood-sugar
19. v. 32	0·1	15 mins.	−	6·855		
,,	,,	,,	−	6·982	74	6·931
,,	,,	,,	−	6·955		
,,	,,	45 mins.	−	6·977		
,,	,,	,,	−	6·931	39	6·954
,,	0·1 + 2·0	2·0 units given 3½ hrs. after 0·1 unit; killed after 4½ hrs.	+	6·923	26	6·934
,,	,,	,,	+	6·923		
,,	,,	,,	+	6·957		

was found to be the case for the tissue under investigation. No doubt, therefore, some change takes place rapidly in the tissue, immediately after excision, which renders it much more acid than it is during life. This does not necessarily invalidate deductions drawn from differences observed in the p_H of brains of animals which have been subjected to varying experimental treatment, and the results of the experiments do, in fact, fall into line with the previous observations on the behaviour of the brain-lactic acid. It is especially interesting that the values for frozen hypoglycaemic brains are actually higher than those for frozen normal ones, and this may possibly indicate that there is some actual p_H difference during life.

The actual decrease in p_H observed on incubation of the brains is shown in the following summary:

	Frozen	Incubated	Difference
Hypoglycaemic	7·029	6·934	0·095
Normal	6·897	6·704	0·193
Hyperglycaemic	6·975	6·444	0·531

It has previously been shown [Holmes and Sherif, 1932] that there is a rise in lactic acid on incubation of the brains, and that this rise is least with hypoglycaemic, greater with normal, and greatest with hyperglycaemic animals. It is clearly of interest to discover whether these changes in lactic acid content of the brain, which are known to occur, would, in fact, give rise to changes in p_H of the order here observed. Figures are available, both from the experiments already reported and from others performed since, for the increase in lactic acid to be expected in each of the three sets of conditions. In addition, the change in p_H to be expected from a given increase in lactic acid has been determined in the following way. The brains of 3 mice, weighing together approximately 1 g., were suspended in 10 cc. of unbuffered Ringer's solution, and the p_H was determined. 0·01 *N* lactic acid was added to the suspension in amounts of 0·05 cc. at a time, from a micro-burette, and the p_H determined after each addition. Titration curves were constructed, in this

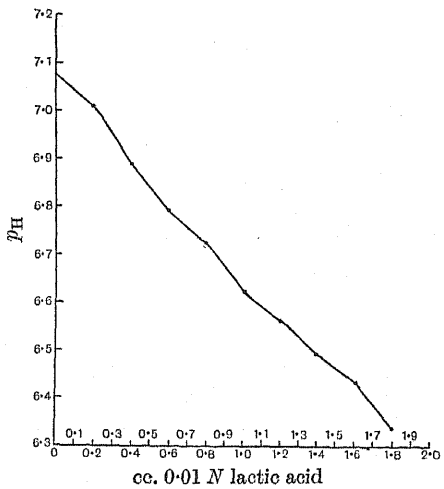


Fig. 1.

way, for the brains of normal and hypoglycaemic animals, both after fixing in liquid air and after incubation. As was to be expected, the initial p_H differed in the different cases, so that the curves in various experiments did not coincide. They were, however, very nearly parallel with each other. Fig. 1 is a curve constructed from the mean values of six of these titration curves. Table II shows the expected increase in lactic acid, the observed fall in p_H, and the fall in p_H read off from the titration curve.

Table II.

	Increase in lactic acid mg. per g. tissue	Increase as cc. 0·01 *N* acid	Fall of p_H (from curve)	Fall of p_H (observed)
Hypoglycaemic	0·16	0·177	0·093	0·095
Normal	0·54	0·60	0·257	0·193
Hyperglycaemic	1·39	1·54	0·640	0·531

For convenience, the increases in lactic acid are calculated in terms of cc. of 0·01 *N* acid per g. of tissue.

In the case of the hypoglycaemic animals, the agreement is good; the rise in lactic acid is small, but the relevant part of the titration curve (p_H 7·029 onwards) is steep. In the other two cases, all that can be said is that the changes observed and the changes calculated are of the same order.

Summary.

The p_H values, determined *in vitro*, of mouse brains which have been fixed in liquid air are higher in the case of hypoglycaemic animals than of normal or hyperglycaemic ones. In all cases the p_H of incubated brains is lower than that of those which are frozen immediately.

The changes in p_H observed are of the same order as those calculated from the titration curve of brain tissue and lactic acid, allowance being made for the amount of lactic acid which would be liberated in the various circumstances considered.

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CCXL. FURTHER OBSERVATIONS ON THE VOLATILE CHLORINE OF BLOOD.

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In a previous paper [Morris and Morris, 1930] it was shown that drying of blood at 105° for a period of 4 hours led to a diminution in the chlorine content and it was suggested that the chlorine lost by drying was probably contained in a volatile organic compound. Sunderman and Williams [1931] published figures indicating that while a loss of chlorine occurred on drying blood and tissues it could be prevented by adding water to the dried blood and allowing the mixture to remain for 3 hours prior to the estimation of the chlorine. They further showed that the addition of olive oil to a solution of sodium chloride with subsequent drying led to the loss of a certain amount of chlorine which also could be prevented by the addition of water after drying and prior to the estimation, and concluded that in the case of blood the loss of chlorine was only an apparent one, being probably due to the effect of occlusion of the chlorine by fat. Quagliariello and Mazza [1931], on the other hand, state that the loss of chlorine on the heating of blood is due to drying and loss of HCl and is therefore no indication of the presence of organic chlorine compounds. Hogartz [1931] found no evidence of organic compounds of chlorine by extraction methods involving the use of alcohol and ether.

In our previous paper the results of experiments were given showing that the chlorine lost from the dried blood could be recovered almost quantitatively although not in the form of free chlorine or hydrochloric acid. In view of the results of Sunderman and Williams with olive oil, it was thought necessary to confirm our previous results and study the variations in the amounts of chlorine volatilised under different conditions.

Effect of drying blood at 105°.

Each sample of blood was divided into three equal parts, and the Cl content of the first was determined immediately by the Van Slyke method. The remaining two were dried for 4 hours at 105°; thereafter one was analysed by

the Van Slyke method and the other was completely ashed and its Cl content estimated by the same method. Table I indicates that practically the same diminution was obtained by drying whether the Van Slyke method was used directly or preliminary ashing was employed. It seems clear therefore that the decrease in the Cl value was not the result of occlusion.

Table I.

Chlorine milliequiv./litre.

Blood no.	No preliminary drying	Preliminary drying	Preliminary drying and ashing
35	89·00	71·90	68·50
36	89·60	71·90	72·30
37	87·10	77·00	76·00

Effect of oxygen on the volatilisation of the chlorine from blood.

To study the effect of oxygen, samples of the same blood were dried in ordinary air, air deprived of oxygen and in an atmosphere of pure nitrogen. The air was deprived of oxygen by passing it through two flasks of pyrogallol prior to its entry into the drying flask. The atmosphere of nitrogen was supplied by heating NH_4NO_2. Table II shows that in the absence of oxygen no loss of chlorine is produced by drying at 105°. Furthermore, in the presence of air, addition to the blood of a strong oxidising agent such as potassium persulphate leads to an increased loss of chlorine on drying (Table III). This suggests that the presence of active oxygen facilitates the formation of the volatile chlorine compound.

Table II.

	No drying	Preliminary drying			
Blood no.		Ordinary air	Dry air	Dry air deprived of oxygen	Nitrogen
46	86·2	72·4	72·8	86·2	86·0
47	79·8	70·1	70·0	78·6	79·1
48	84·5	69·9	70·4	82·9	84·8
49	88·9	74·5	73·9	87·9	88·3
50	79·8	64·8	64·8	78·7	79·6
51	86·5	75·6	75·5	86·0	86·1

Table III. *Effect of addition of potassium persulphate to blood on the chlorine content after drying.*

	No drying		Preliminary drying	
Blood no.	Blood	Blood + $K_2S_2O_8$	Blood	Blood + $K_2S_2O_8$
51	85·66	85·60	73·00	62·30
52	85·20	85·25	69·95	59·24
53	87·68	87·72	65·36	57·71
54 (serum)	115·85	115·81	114·32	103·61

Effect of drying blood in a desiccator (Table IV).

Sunderman and Williams showed that drying blood over P_2O_5 also involved a loss of Cl and concluded that the essential factor was the loss of moisture. We studied the effect of drying blood for 48 hours over H_2SO_4: from some flasks oxygen was removed, others were filled with nitrogen, while others contained sodium cyanide solution. The effect of drying over H_2SO_4 was to produce a

Table IV. *Effect of drying blood in desiccator at room temperature (18°) in atmospheres of* (a) *air*, (b) *air deprived of oxygen*, (c) *nitrogen*, (d) *air, with NaCN added to the blood and* (e) *air, with $K_2S_2O_8$ added to the blood.*

Blood no.	No drying	Preliminary drying in oven at 105°	Air	Air deprived of oxygen	Nitrogen	Ordinary air; NaCN added to blood	Ordinary air; $K_2S_2O_8$ added to blood
(*a*) 46	86·2	72·4	78·8	86·2	86·0	85·5	71·1
(*b*) 47	79·8	70·1	74·9	79·5	79·8	79·8	71·9
(*c*) 48	84·5	69·9	74·9	84·0	84·1	84·0	67·3
(*d*) 49	88·9	74·5	80·6	89·0	87·9	87·8	76·0
(*e*) 50	79·8	64·8	71·4	78·8	78·9	78·4	60·6

diminution in the amount of chlorine recovered, although the loss was not so great as when the blood was dried at 105°. The addition of potassium persulphate increased the loss to at least that found on drying at 105°. The absence of oxygen from the flask or the presence of cyanide, which inhibits oxidation, prevented any loss of chlorine. It is clear therefore that the withdrawal of moisture in itself is not responsible for the diminution in chlorine content. It should here be stated that if the cyanide-blood mixtures are dried for over 48 hours the cyanide is converted into HCN and completely volatilised and there may occur some oxidative process with consequent loss of chlorine (Table V).

Table V. *Effect of drying blood with NaCN in desiccator for varying periods.*

		Preliminary drying		
Blood no.	No drying	Duration of drying in hours	Blood alone	Blood + NaCN
46	86·2	24	82·4	85·9
—	—	48	80·6	86·0
—	—	72	72·4	85·5
—	—	120	72·9	80·6
—	—	168	72·1	72·8

Table VI. *Effect of drying tissues* (a) *at 105°*, (b) *in desiccator and* (c) *with acetone and ether.*

Tissue (rabbit)	No drying	In oven at 105°	In desiccator	By acetone and ether
Heart	35·4	27·5	32·6	35·8
,,	33·8	26·2	30·1	35·0
Kidney	49·2	31·8	40·4	48·8
,,	54·1	36·5	46·2	54·0
Muscle	12·2	8·1	10·4	12·2
,,	16·4	11·9	14·0	16·0
,,	10·5	7·0	8·8	10·5

Effect of drying tissues under various conditions.

Prior to determination of their Cl content tissues were dried either in the oven at 105° or over concentrated H_2SO_4 or by Best and McHenry's acetone and ether method [1930]. The results (Table VI) fully corroborate the blood analyses, indicating that oxygen is essential for the volatilisation of the chlorine and that more chlorine is lost at a temperature of 105° than as a result of drying in a desiccator.

Effect of addition of olive oil to NaCl solution.

It seemed to us possible that the loss of chlorine on drying a mixture of olive oil and NaCl [Sunderman and Williams, 1931] might be due to reaction between the NaCl and unsaturated fatty acids.

This view was supported by the fact that heat is necessary to produce a loss of Cl from the olive oil-NaCl mixture. Further, the use of a fully saturated acid such as palmitic instead of olive oil should lead to no diminution of Cl content on drying the fatty acid-NaCl mixture at 105°. Experiments show that heating of palmitic acid-NaCl mixtures at 105° leads to no decrease in the Cl.

The only difference between blood and the olive oil-NaCl mixture as far as the loss of chlorine is concerned is the fact that the chlorine is lost from the latter mixture only after heating to over 100° whereas it can be driven off from the blood on drying in the desiccator without the aid of heat. This, however, is insufficient evidence that chlorine is present as an organic compound in blood. In order to demonstrate the presence of such a compound in blood it is necessary to extract it.

Organic solvents such as ether, benzene and toluene were found to be useless for the extraction of any chlorine. It was thought that a mixture of alcohol and ether might be efficient. Alcohol, however, mixed with the water present in blood and carried into solution some of the inorganic chlorine. It was therefore necessary to remove water from the blood by some method other than heat. For this purpose a modification of Best and McHenry's method for drying tissues was used. This consisted in the addition of acetone to blood, filtering, and washing four times with acetone in excess until no chlorine appeared in the acetone filtrate and then three or four times with ether. Air was then drawn over the residue by a suction pump, until a dry powder was obtained. The powder was removed to a flask and a mixture of equal parts of absolute alcohol and anhydrous ether was added. The powder was shaken with the alcohol-ether mixture for three periods of 15 minutes and then allowed to stand overnight. The alcohol-ether extract was then filtered off into a pyrex tube, and the alcohol and ether were evaporated off at a low temperature. Chlorine was invariably found to be present in the extract when this was treated with silver nitrate and nitric acid. So far we have been unable to recover more than 80 % of the amount lost on heating blood. With tissues the same method was employed after they were finely minced.

The alcohol-ether mixture does not extract chlorine from inorganic chlorides unless water is present. This we have repeatedly confirmed for the alcohol-ether mixtures we have been using.

It seemed possible, however, that the blood might have been incompletely de-hydrated by the acetone-ether treatment, and that the chlorine extracted was due simply to the inorganic chlorides being dissolved in the alcohol-ether mixture owing to the presence of moisture. To test this possibility a series of experiments was undertaken in which a synthetic mixture analogous to blood was used, containing 16 g. of protein (egg-albumin, serum-albumin or caseinogen), 0·5 g. of olive oil, 0·5 g. of NaCl and 0·1 g. of glucose per 100 cc. water. The mixture was well stirred and allowed to stand for about 4 hours prior to extraction, by the method used for the blood. In no case was chlorine found in the alcohol-ether extract.

Further, it was shown in a previous paper [Morris and Morris, 1930] that the volatile chlorine of blood diminishes on adding acids to the blood, the diminution being dependent on the strength of the acid. The addition of a strong acid should therefore diminish the amount of organic chlorine extracted from blood by means of alcohol and ether. From the same sample of blood two lots of 100 cc. were taken to one of which was added 10 cc. concentrated HCl. Both were allowed to stand for 4 hours and then extracted by acetone and finally by alcohol and ether. In the blood-HCl sample only minimum traces of chlorine (1–2 milliequiv.) could be detected in the alcohol-ether extract. This experiment which was repeated on four occasions affords further confirmation of the fact that inorganic chlorine is not extracted by the method described.

In view of these facts it seems justifiable to conclude that there exists in blood an organic chlorine compound.

Summary.

1. The presence of oxygen is essential for the loss of chlorine to take place.

2. The heating of an olive oil-NaCl mixture at a temperature over 100° leads to loss of chlorine.

3. Evidence is brought to show that there is close similarity between the two systems, blood and olive oil-NaCl, as far as the loss of Cl is concerned, except that in the latter mixture heat is required whereas with blood drying in a desiccator is sufficient to cause volatilisation of the Cl complex.

4. A method is described whereby organic chlorine can be extracted from blood. The method depends on removal of water by acetone and ether and subsequent extraction of the chlorine by an alcohol-ether mixture.

The authors regret that owing to an unfortunate oversight there was omitted from their previous paper an acknowledgment that the work therein described was commenced by one of them (S. M.) while in the Chemical Department of the Pennsylvania Hospital, Philadelphia.

We wish to express our thanks to Prof. G. G. Henderson, Prof. G. B. Fleming and Dr N. C. Wright for their helpful criticism and to the Medical Research Council for defraying the expenses of the investigation and for a personal grant to one of us (N. M.).

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CCXLI. FAT METABOLISM OF THE HERRING.
I. A PRELIMINARY SURVEY.

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The gross chemical changes which occur in the tissues of the herring in different phases of sexual maturity have been studied by a number of workers. Milroy [1906; 1908] in a study of the muscles and gonads of Loch Fyne herrings observed a rapid rise in the percentage of fat (ethereal extract of tissue dried at 60–70°) in the muscles during the several months prior to the time when rapid growth of ovaries and testes began. This rise was followed by a steady fall and the lowest percentage was reached after the fish had spawned some 3 months later. The percentage of fat in the ovaries was variable. Similar observations were made on fish from the East Coast of Scotland [Milroy, 1907]. In these studies sexual maturity, length and weight of the fish were considered. Johnstone [1920] as a result of successive annual investigations [Johnstone, 1915; 1918; 1919] of the muscle-fat (carbon tetrachloride extract of tissue dried at 100° for 24 hours) was able to construct curves showing that the fat content of the muscles of Manx herring, which was less than 5 % in May, rose to a maximum in July or August (30–35 %), and then fell rapidly after the fish had spawned. The shape of each curve was characteristic of each season. Bruce [1924] deduced that, at any given stage of sexual maturity, the fat content of herring muscle was increased by age. He also quotes some results of Secker on the amount of fat in the livers and gonads of a number of samples of Clyde herring.

Thus, although much is known of the fat content of various tissues of the herring at different seasons, investigations have hitherto been mainly concerned with the herring as food. They have established irrefutably the progressive rises and falls in the fat content of the edible portion of the fish, but in no case have the investigations been comprehensive enough to allow of deductions being made as to the sequence of changes in fatty constituents occurring in the different tissues of a fish of given age throughout the period of gonad growth.

The work described in this paper was undertaken in an attempt to obtain more exact information regarding the rise and fall in the amount of the fatty constituents of the different tissues from the time when the gonads were

empty through the various stages of gonad development until the fish had spawned. By studying the amounts of fatty acids and cholesterol of all the tissues throughout the season, it was hoped to throw some light on the processes of translocation of these substances to the gonads.

Apart from the muscle and gonads, therefore, the livers have also been studied. Further, in the herring there is present at certain stages of the season a quantity of fat attached to the outside of the alimentary tract. This "mesenteric" fat has also been included in the investigations and, for ease of description in the Tables, this fat has been described as belonging to the intestine.

Collection of samples.

Samples of fish were collected once weekly over a period of 14 weeks from June 9 to September 11, 1931, from the marketable catches landed at Port Erin and Peel. From the average catch, something like 60 fish were taken as a sample. From the latter number, after investigation of length, sex and state of maturity, two further samples were taken to represent as far as possible the average of all these factors, each sample consisting of 6 male and 6 female fish. The region from which the fish were taken lies within an area bounded by a line drawn parallel to the west and south-west coasts of the Isle of Man between Peel and Port St Mary, and 8 to 10 miles from the coast, which constitutes the Port Erin fishing ground.

Observations of length, sex, state of maturity of roe or milt on Hjort's scale, and age were made on each fish of every sample. The number of vertebrae in the whole samples (about 1600 fish) were counted. These results are published separately [Saby, 1932]. Length was measured from the snout tip to the end of the longest caudal fin ray in the dorsal fluke of the tail.

The degree of maturity was classified on Hjort's scale. The classes actually encountered were: I, virgin, II, maturing virgins or recovering spents; III, gonads filling one-half the body cavity; IV, gonads filling more than two-thirds of the cavity; V, full; gonads filling the body cavity; VII, spent.

For the determination of age, several scales from the anterior region near the tip of the pectoral fin were removed and dried in air. Each collection was preserved in a numbered envelope, and later the scales were cleared and the winter rings counted under a binocular microscope according to the method of Lea [1910].

After determination of its weight, each fish was carefully dissected, and the liver, gonad and alimentary canal and a portion of the muscle were removed. The livers and gonads were readily obtained, but it was not found possible to remove the alimentary tract without some loss of the adherent fat. The alimentary tract was severed at the oesophagus and taken out entirely. The stomach was then opened and cleared, and gentle pressure was applied to the intestine in order to squeeze out as much of the remaining food residues as possible. The muscle sample was taken by cutting a fillet of the edible flesh extending from just behind the pectoral fin to the tail region. The selected tissues were then stored in stoppered bottles under excess of acetone until the collection was completed (14 weeks), when they were brought to Liverpool and worked up during the following 8 months. Each week there were thus obtained bottles containing respectively the livers, gonads, alimentary tracts and muscle fillets from 6 male fish, and the corresponding samples from 6 female fish. Table I gives the data concerning all the samples.

The determination of fatty acids and the unsaponifiable fraction.

Because of the labour and expense involved, it was decided in the first place to work up the samples from alternate weeks; the results obtained were so definite as to make it unnecessary to investigate the remainder.

The acetone was removed from each sample by filtration and the residue was repeatedly warmed with fresh samples of acetone, filtration and grinding of the residue in a mortar following each extraction. The combined acetone filtrates were evaporated to dryness *in vacuo*. The dry powdered residue was then extracted with ether (Soxhlet, 8 hours). The ethereal extract was added to the residue from the acetone, and the whole evaporated to dryness. The residue obtained

Table I.

Date	Sex	Length (cm.) 21	22	23	24	25	26	27	28	29	Age (years) 2	3	4	5	6	7	8	Maturity (Hjort's scale) I	II	III	IV	V	VII	Wt. of the 6 fish g.	Wt. of livers g.	Wt. of gonads g.	Wt. of alimentary tract g.
June 9	♂	.	1	2	2	1	.	.	.	.	3	3	.	.	.	.	.	3	3	.	.	.	.	598	11·0	2·5	23·0
	♀	.	1	2	3	.	.	.	.	.	4	2	.	.	.	.	.	2	3	1	.	.	.	574	11·0	6·5	24·0
„ 17	♂	.	1	1	2	1	1	.	.	.	1	4	1	.	.	.	.	1	3	2	.	.	.	710	13·0	10·5	36·0
	♀	1	1	1	1	1	1	.	.	.	2	3	1	.	.	.	.	1	2	3	.	.	.	659	16·4	17·1	31·1
July 7	♂	.	1	1	2	1	1	.	.	.	2	2	2	.	.	.	.	1	.	3	2	.	.	777	10·7	27·0	42·0
	♀	.	1	2	1	1	1	.	.	.	3	2	1	.	.	.	.	.	1	4	1	.	.	760	14·5	22·4	40·0
„ 24	♂	.	.	2	2	1	1	.	.	.	2	3	1	.	.	.	.	.	.	.	2	4	.	848	9·0	91·0	26·5
	♀	.	.	1	2	2	1	.	.	.	1	4	1	.	.	.	.	.	.	2	1	3	.	831	17·7	57·0	30·0
Aug. 5	♂	.	.	1	2	2	1	.	.	.	1	3	2	.	.	.	.	.	.	1	.	5	.	888	9·0	128·0	22·5
	♀	.	.	1	3	1	1	.	.	.	2	2	2	.	.	.	.	.	.	1	1	4	.	841	16·0	63·0	25·0
„ 20	♂	.	.	1	2	1	1	1	.	.	1	2	3	.	.	.	.	.	.	.	.	6	.	873	5·5	158·0	13·0
	♀	1	.	.	2	1	1	1	.	.	1	3	1	1	.	.	.	.	.	.	1	5	.	866	13·6	106·0	16·0
Sept. 5	♂	.	.	.	.	.	.	3	3	.	.	.	2	2	1	1	.	.	.	.	.	6	.	1162	5·5	256·0	12·0
	♀	.	.	.	.	.	1	4	1	.	.	1	1	3	.	.	1	.	.	.	.	6	.	1102	16·0	212·0	15·0
„ 11	♂	.	.	.	.	.	2	.	3	1	.	.	1	4	1	.	.	.	.	.	.		6	915	12·0	Spent	11·0
	♀	.	.	.	.	.	1	3	.	1	.	.	1	3	1	.	1?	.	.	.	.	.	6	850	11·0	„	14·0

was then saponified by boiling with excess of alcoholic potassium hydroxide and, after removal of much of the alcohol, followed by dilution with water, the unsaponifiable fraction was obtained by 6 extractions with ether. The ethereal extract, after washing with water to neutrality, yielded, on evaporation and drying *in vacuo* at 100°, the unsaponifiable fraction. The combined soap solutions and aqueous washings were acidified with hydrochloric acid, and similarly extracted with ether. After washing, the ethereal extract was evaporated and the residue dried at 100° *in vacuo*. It was then dissolved in light petroleum and allowed to stand overnight. After filtration, the light petroleum solution yielded, on evaporation, the fatty acids, which were dried at 100° *in vacuo* and weighed. Iodine values [Rosenmund and Kuhnhenn, 1923] were determined on the fatty acids and unsaponifiable fraction, and the amount of cholesterol in the latter by the digitonin method.

Results.

The percentage yield of fatty acids.

The yields of fatty acids (g. per 100 g. of fresh tissue) are recorded in Table II.

Table II. *Percentage yield of fatty acids.*

	Liver		Intestine		Muscle		Gonad	
Date	♂	♀	♂	♀	♂	♀	♂	♀
June 17	11·43	10·76	30·85	22·32	10·00	10·66	5·14	2·61
July 7	14·48	15·47	40·34	38·30	13·71	17·19	5·64	4·79
„ 24	9·31	8·83	31·93	28·99	17·83	17·09	1·82	3·84
Aug. 5	13·12	10·92	23·59	21·10	17·54	14·58	1·58	4·35
„ 20	5·13	5·77	10·63	15·31	15·09	16·04	1·90	3·16
Sept. 5	2·59	3·92	1·47	2·85	12·73	14·24	2·12	4·15
„ 11	0·99	1·29	0·87	1·26	10·86	Lost	Spent	Spent

These figures show that in the 21 days between June 17 and July 7 there has been a very large increase in the percentage of fatty acids in the liver, intestine and muscle. The percentage increases over the values of June 17 are as follows: liver ♂ 27·0, ♀ 43·8; intestine ♂ 30·8, ♀ 71·6; muscle ♂ 37·1, ♀ 61·3. In the liver and intestine, both for male and female fish, July 7 marks the highest values obtained throughout the experiment. From this date onwards there is a steady decrease throughout the remainder of the period. Thus, in the last samples, the spent fish of September 11, the percentage of fatty acid in the livers has sunk to 0·99 and 1·29 for the males and females respectively, as against 14·48 and 15·47 % on July 7, showing that just over 90 % of the initial liver-fatty acids have been utilised or translocated elsewhere. The results with the intestinal fat are even more striking, for the fatty acids disappearing account for 97·9 and 96·7 % of those present on that date. The maximum percentage of fatty acids in the muscle appears to occur rather later, July 24, and the final percentage in the last sample in the male fish shows a fall of 39 % from the highest value. The final female sample was unfortunately lost; but the value for the previous collection of September 5 shows a fall of 17·2 %. The percentage fatty acid content of the gonads in the different samples shows no regular change, which is perhaps a somewhat unexpected result, although similar to that of Milroy [1908].

The results are expressed graphically in Fig. 1 where the marked initial rises in the percentage fatty acids of liver, intestine and muscle are clearly

seen, as are the gradual progressive losses of fatty acids from these tissues with gonad development. The approximately 70 % increase in the percentage of muscle-fatty acids in the first 38 days is noteworthy. (It is to be observed that in Fig. 1 the curves are all drawn on the same scale save those for the intestinal fat which, reaching a percentage of 40 and falling finally to about 1 %, necessitates a scale one-fifth as great.)

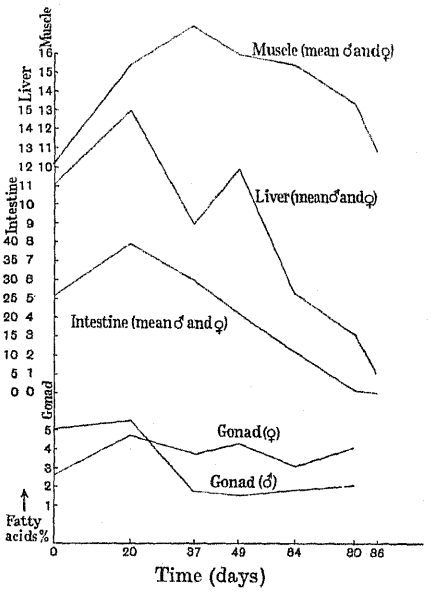


Fig. 1. Variation in the percentage of fatty acids in the tissues of the herring during the maturation period.

The percentage yield of unsaponifiable matter.

The yields of unsaponifiable matter (g. per 100 g. of fresh tissue) are recorded in Table III.

Table III.

	Liver		Intestine		Muscle		Gonad	
Date	♂	♀	♂	♀	♂	♀	♂	♀
June 17	1·09	0·81	0·54	0·61	0·19	0·19	0·43	0·47
July 7	1·04	0·94	0·58	0·61	0·15	0·15	0·63	0·52
,, 24	1·11	0·72	0·76	0·65	0·15	0·22	0·38	0·58
Aug. 5	1·13	0·67	0·60	0·45	0·17	0·18	0·45	0·54
,, 20	0·72	0·49	0·71	0·67	0·19	0·22	0·60	0·47
Sept. 5	1·40	0·41	0·92	0·66	0·13	0·16	0·66	0·51
,, 11	0·31	0·46	0·37	0·48	0·15	—	Spent	Spent

In Table IV the unsaponifiable matter is expressed as a percentage of the fatty acids. The unsaponifiable fractions consist largely of cholesterol, the average figures being liver 42 %, intestine 57 %, muscle 37 %, gonad 84·5 %.

Table IV.

Date	Liver ♂	Liver ♀	Intestine ♂	Intestine ♀	Muscle ♂	Muscle ♀	Gonad ♂	Gonad ♀
June 17	9·6	7·5	1·75	2·74	1·9	1·78	8·3	18·0
July 7	7·2	6·1	1·44	1·59	1·09	0·87	11·2	10·9
„ 24	11·9	8·2	2·38	2·24	0·84	1·29	20·9	15·1
Aug. 5	8·6	6·1	2·54	2·13	0·97	1·23	28·5	12·4
„ 20	14·0	8·7	6·68	4·37	1·26	1·37	31·6	14·9
Sept. 5	54·0	10·5	62·60	23·12	1·02	1·14	31·1	12·3
„ 11	31·3	35·7	42·53	38·09	1·38	—	Spent	Spent

From the figures recorded in Table IV it is clear that the rate of utilisation of the unsaponifiable fractions is very much less rapid than that of the fatty acids of the liver and intestine, which Table II shows are almost completely lost from those tissues by the time that maturation is complete. Thus, whereas in the liver on June 17 the percentage of unsaponifiable matter is about 1/12 that of the fatty acids, on September 11 it constitutes about 1/3, a fourfold increase. Similarly for the intestine, the ratio of unsaponifiable matter to fatty acids rises from its initial value to a final one something like 20 times as great. The high ratio in the gonads is to be expected, while the lack of regularity in these figures is consequent upon the variable percentages of fatty acids in the different samples, as recorded in Table II.

The iodine values of the fatty acids.

It is not necessary to reproduce the iodine values in full, because, save in the case of the gonads, the variations obtained were irregular and no deductions could be made from them. The extreme and mean values are recorded in Table V.

Table V. *Variations in iodine values of the fatty acids.*

	Highest ♂	Highest ♀	Lowest ♂	Lowest ♀	Mean of all ♂	Mean of all ♀
Liver	133	141	120	115	127	130
Intestine	124	122	91	91	107	109
Muscle	136	126	102	105	116	119

In the case of the gonads, however, the fatty acids showed a progressive increase in unsaturation with the development of maturity, and these figures are presented in Table VI.

Table VI. *Iodine values of fatty acids of gonads.*

	June 17	July 7	July 24	Aug. 5	Aug. 20	Sept. 5	Sept. 11
♂	128	151	162	158	195	209	Spent
♀	146	143	163	170	174	195	„

The high degree of unsaturation of the fatty acids just before spawning is noteworthy, as is also the fact that these fatty acids are very much more unsaturated than even those of the liver itself.

Superficially, the fact that the gonad-fatty acids have iodine values some 50 % greater than the fatty acids of any of the other tissues suggests that desaturation of the fatty acids is occurring, especially as for the last month at least the fish is not feeding. Alternatively, the high degree of unsaturation of the gonad-fatty acids might be due to preferential translocation of the more unsaturated acids from the liver. The first point to be mentioned, as will be shown later, is that at the time of maturation the gonads contain about 1 g. of total fatty acid, whereas the liver has a maximum fatty acid content of only about 0·3 g. which falls to about 20 mg. when maturation is complete. Since the iodine value of the liver-acids does not change significantly during the maturation period, and further, since the total loss of fatty acid from the liver is no more than 0·28 g., it is clear that the fatty acids which the liver has lost cannot in any way explain the presence in the gonad of 1 g. of fatty acid of iodine value 200. On the other hand, preferential transference of the more unsaturated acids from the muscle to the gonad could account for the high iodine value of the gonad-fatty acids, for the muscle of the average fish contains about 17 g. of fatty acids during the earlier period, of which about 4 g. are lost by the time that maturation is complete. These 4 g. of iodine value 116 would provide 464 available iodine units, of which the 1 g. of fatty acids in the gonads at maturity require only 200. Hence the loss of fatty acids from the muscle could account for the appearance of 1 g. of fatty acids of iodine value 200 in the ripe gonad, without the iodine value of the remaining muscle-fatty acids changing. Similar reasoning can be applied to the fatty acids of the intestinal fat. During the later stages of the maturation period the fish is calling on its reserves of fat for energy purposes. If the more unsaturated acids disappearing were translocated to the gonads and only the more saturated acids were being burnt, then results recorded in Table VI would be explained without invoking the aid of a desaturation hypothesis. On the other hand, it is possible that the gonad-fatty acids may represent the results of a desaturation process occurring in the liver or elsewhere, and evidence on this question is now being sought. Further, the possibility of a synthesis *in situ* must not be overlooked. The occurrence of these acids of high iodine value in the ovaries and testes may suggest that they are present as phosphatides, which the findings of Milroy [1906; 1908] and Bruce [1924] of an increasing phosphorus content in these tissues with maturation may imply.

Yield of sterol.

Cholesterol was not estimated in the unsaponifiable fractions until the samples of July 24. These are set out in Table VII. These figures show a regular decrease in the percentage of liver-cholesterol, while that of the muscle remains constant. The figures for the cholesterol content of the intestine are somewhat irregular.

On the other hand the contrasting results for the testes and ovaries are

worthy of mention. Whereas in the testes the amount of cholesterol is steadily increasing to a maximum prior to the spawning of the fish that of the ovaries shows but small and irregular changes with the approach of maturity.

Table VII. *Yield of sterol per 100 g. of fresh tissue (mg.).*

	Liver		Intestine		Muscle		Gonad	
Date	♂	♀	♂	♀	♂	♀	♂	♀
July 24	—	431	294	338	67	75	335	458
Aug. 5	559	366	316	351	68	65	369	438
,, 20	375	261	—	318	64	69	533	397
Sept. 5	263	187	492	297	—	66	615	410
,, 11	168	197	244	—	66	—	Spent	Spent

The absolute gains and losses of fatty acids and cholesterol by the tissues.

With tissues which are increasing or decreasing so rapidly in weight (gonad, liver, intestine), the expression of the fatty constituents as percentage figures gives no idea of the changes in weight which these constituents undergo. It is of considerable interest therefore to attempt to convert these percentage figures into weights, in order to gain some idea of the magnitude of the loss or gain of the fatty constituents by the various tissues in the successive stages of maturation. This raises the question as to whether it is safe to assume for the purposes of discussion of the results that fish drawn from one fishing ground over a limited time, if comparable as regards age and sexual maturity, may be regarded within rough limits as comparable in other respects, provided the inherent error of small random sampling be adequately recognised at the outset. It is of course definitely known that herring spawn at definite and often widely differing times in different latitudes. Thus herring in the Port Erin area always spawn in August and September, while in Western Scottish waters the period is January and February [Milroy, 1908], and other examples may be cited. Unless therefore the herring caught in a given area consist of a number of different races, in which the weight to age ratio varies considerably, it appears to us that it may not be unprofitable to compare the weights of the fatty constituents present in successive samples and to consider the changes in these weights as roughly representing the results of the metabolism during the intervening periods, provided the ages of the fish and the degree of sexual maturity which are known are comparable. (The results of counting the vertebrae of the 1600 fish from which our samples were drawn provide no evidence for the existence of different races [Saby, 1932].)

Fourteen samples in all were analysed over the period June 17 to September 11, each sample consisting of 6 fish, and we may now consider the ages of the fish constituting the different samples. It is seen (Table I) that in every one of the 10 samples from June 17 to August 20 there are present one 2-year old, two 3-year old and one 4-year old fish, *i.e.* in each sample 4 of the 6 fish are of the same age. Of the 60 fish, therefore, 40 are equally distributed as regards age in the 10 samples. Further, save for one

individual fish, the remaining 20 are also 2, 3 or 4 years old. We may take it also that any 3-year old fish in these 20 should play no part in rendering the weight relations incomparable. Of these there are 8. Hence it seems to us that of the 60 fish in the 10 samples only 12 should cause any possible serious variation in weight relationships. Further, in two samples there are two 2-year old fish, but these are offset by the presence of two 4-year old fish, and these samples also should therefore be comparable with the rest. Thus, of the 60 fish involved in these 10 samples, only 8 should cause any appreciable variation and, remembering that the difference in size of 2, 3 and 4-year old herring is not large, it seems to us that these 10 samples may be compared as a rough approximation. Further, the average length of the 6 fish in each of these 10 samples is 24, 23·5; 24·0, 23·8; 24·2, 24·5; 24·5, 24·3; 24·8 and 24·7 cm. over 65 days. This appears to confirm the reasoning regarding the even distribution as regards ages, and strengthens the ground for regarding the weight of fatty acids in the tissues of the fish of various successive samples as representing actual metabolic changes occurring between successive samplings. These samples are also in order of increasing maturity (Table I).

On the other hand, the last four samples of September 5 and 11 are not comparable with these 10, because, of the 24 fish involved, 18 are over 4 years old. The average ages of these samples are 5·3, 5·0, 6·0 and 5·2 years, and their average lengths 27·5, 27·5, 27·5 and 27·3 cm., whereas the extremes in the average length of the fish in each of the first 14 samples are 23·5 and 24·8 cm.

Although the weights on September 5 and 11 are thus vitiated it is of some importance to derive figures for these two dates, especially for September 5, which was the last day on which the fish were taken before spawning, and we think that a close approximation can be obtained.

Before proceeding to discuss this matter further it is to be pointed out that only fillets of the muscle were taken and hence the total muscles of each fish were not weighed at the time of collection, but the approximate weights of the muscles can be derived from Table I. In order to obtain a figure for the muscles of the herring, 8 fish of markedly varying weights and gonad development were dissected and the alimentary tracts, livers and gonads removed. The residue, consisting of muscle, head, tail and skeleton was weighed, W. The muscle was then torn away and the head cut off. The combined weight of the head and tail and the skeleton after washing in water and roughly drying represented 17·6 to 21·3 % of W. For the purposes of this discussion, therefore, the total weight of muscle may be taken as 80 % of the weight of the total fish less that of the alimentary tract, liver and gonad. Using this figure of 80 %, therefore, the figures on p. 2030 are thus derived from Table I.

The big increases in the muscle weight of the samples of September 5 and 11 are at once apparent.

We incline to the view that the percentage composition of fish in the same stage of maturity does not vary significantly with age. It is true that Bruce

	June 17		July 7		July 24		Aug. 5	
	♂	♀	♂	♀	♂	♀	♂	♀
Wt. of muscle	86·8	84·0	93·0	91·1	96·2	97·2	97·1	98·1

	Aug. 20		Sept. 5		Sept. 11	
	♂	♀	♂	♀	♂	♀
Wt. of muscle	92·9	97·3	118·4	114·5	119·0	110·2

[1924] deduces from his muscle analyses that the fat content of the muscle at any given gonad stage depends to some extent on age, but from the present work it is clear that any slight difference due to age is insignificant relatively to the influence of different states of sexual maturity. Now the muscles of the 6 samples preceding September 5 approximate in a sufficiently constant manner to 97 g. to make it accurate for our purposes for this weight to be taken for the samples of September 5 and 11. In Table VIII and Fig. 2 which follow, therefore, the total muscle-fatty constituents for these two dates has been derived by multiplying the found percentages not by the known weights but by the deduced figure 97.

No similar correction need be applied to the liver and intestine, for at this stage their respective weights are only about 2 g. and they contain only about 1 % of fat.

The last gonad weights, however, of September 5 are naturally in need of correction. Since this tissue is increasing in weight rapidly at this time, it is an uncertain proceeding to attempt to correct it, and therefore in the Tables which follow the figures for the gonads of September 5 appear in brackets, and the last portion of the curve is shown as a broken line.

It is of interest to note in passing that the weights of fatty acids in the gonads plotted against time lie on a straight line up to the date preceding September 5. If this straight line were continued to give a corrected figure for the value under discussion we obtain 0·66 g. as against the 1·18 g. given by the enhanced finding on the actual weight of the older fish. That this figure (0·66 g.) must be significantly low is shown by the cholesterol figures in Table IX. Reduction of the sterol figure of September 5 proportionately results in a sterol figure for the September 5 samples actually below that of August 20. This means that, if cholesterol and fatty acids are increasing at the same rate, the fatty acid figure must be considerably greater than 0·66 g.

There are now presented tables showing the variation in the weights of the fatty acids in the different tissues of the average fish.

The figures in Table VIII may therefore be taken as an approximate indication of the changes which occur in a 3-year old herring during the period of active growth of the gonads from an initial weight of 0·8 g. to a final weight of 39 g. when spawning occurs. The liver-fatty acids increase during the first 3 weeks from an initial value of 0·27 g. to the slightly higher figure of 0·31 g. For the remainder of the period, the liver steadily loses its fatty acids until in the week after spawning not more than 20 mg. remain. The intestinal fat

Table VIII. *Total weight of fatty acids in the different tissues of the average fish (g.).*

Date	Liver ♂	Liver ♀	Intestine ♂	Intestine ♀	Muscle ♂	Muscle ♀	Gonad ♂	Gonad ♀
June 17	0·26	0·29	1·85	1·16	8·68	8·95	0·09	0·07
July 7	0·26	0·37	2·82	2·57	12·75	15·66	0·26	0·18
„ 24	0·14	0·26	1·40	1·45	17·16	16·61	0·27	0·36
Aug. 5	0·20	0·29	0·87	0·89	17·03	14·31	0·33	0·46
„ 20	0·05	0·13	0·23	0·41	14·02	15·62	0·50	0·56
Sept. 5	0·02	0·10	0·03	0·08	12·35*	13·82*	(0·90)	(1·47)
„ 11	0·02	0·02	0·02	0·03	10·54*	Lost	—	—

* Corrected as described on p. 2030.

increases similarly in the first 21 days to 2·7 g. and the whole of this amount is then steadily lost by the time that spawning occurs. The fatty acids of the muscle, 8·8 g., however, regularly increase for a rather longer time (about 5 weeks) and thus reach their maximum towards the half-way stage of the maturation process at 16·8 g. They then undergo a roughly similar rate of decrease to a weight of about 10·5 g. in the 13th week. Meanwhile the gonad-fatty acids commencing at 80 mg. increase steadily to a maximum prior to spawning. These rises and falls are illustrated in Fig. 2.

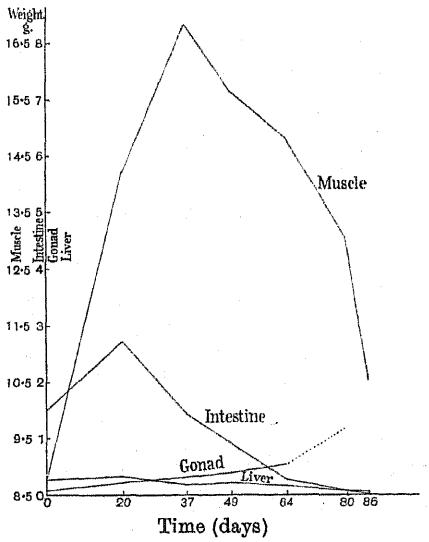


Fig. 2. Variation in weight of fatty acids in the tissues of the average fish during the maturation period (mean ♂ and ♀).

It is not possible to deduce the source of the gonad-fatty acids. It is perhaps of importance to observe that the fatty acids of the liver and mesentery have begun to decrease after 21 days although the muscle-fatty acids are rapidly increasing in the male fish and slightly in the female. This is the more striking when it is remembered that the increase in the muscle-fatty acids

during the next 17 days in the male is 4·4 g., and yet during this time the intestine has lost 1·4 g., and even the very small initial amount of fatty acid in the liver, 0·27 g., is tending to fall. It appears as though the intestinal fatty acids were serving as reserve fat for increasing the muscle-fat as required, but it is not easy to see why fat deposited as reserve fat should be mobilised in order to increase that of the muscles, unless the muscles were possibly playing some part in the preparation of those fatty acids for use possibly by the gonads. It is to be noted that no food was found in the fishes' alimentary tracts after the beginning of August, *i.e.* when the intestinal fat has begun rapidly to decrease. Equally curious also is the fact that the liver containing only 0·27 g. of fatty acid should lose the greater part of this small amount when such relatively vast quantities are available in the muscle.

The losses and gains of the tissues from the time of maximum fatty acid content to September 11 are as follows:

	Fatty acid (g.)	
	Loss	Gain
Liver	0·25	—
Intestine	2·65	—
Muscle	3·76	—
Gonad	—	0·98
Total	6·66	0·98

The net loss of 5·68 g. must have gone towards the supply of energy during the final period of abstention from food.

Table IX. *Total weight of cholesterol in the different tissues of the average fish (mg.).*

	Liver		Intestine		Muscle		Gonad	
Date	♂	♀	♂	♀	♂	♀	♂	♀
July 24	—	12·7	12·9	16·9	64·5	72·9	51	43
Aug. 5	8·4	9·9	11·7	14·7	66·0	63·8	79	46
„ 20	3·3	5·9	—	8·5	59·5	67·1	140	70
Sept. 5	2·4	5·0	9·8	7·4	—	64·0*	(262)	(145)
„ 11	3·4	3·5	4·4	—	64·0*	—	Spent	Spent

* Corrected as described on p. 2030.

From the figures in Table IX it is seen that there is a steady loss of cholesterol from the liver and intestine, and a rapid gain by the gonad of that substance, while the figure for the muscle remains constant. Further, at every stage of maturation the milt contains considerably more cholesterol than the roe.

It seems to us that these figures provide evidence that the fish is synthesising cholesterol. In view of the uncertainty which exists as to the last figure for the gonad-cholesterol on September 5, as discussed on p. 2030, it may be wise to consider that of August 20, which must be very considerably less than the true value for September 5. In the period July 24–August 20 the gonads have gained 56 mg. of cholesterol. In the same period the liver on

the average has lost about 9 mg. and the intestine 6·3 mg., a total of about 15 mg. Bearing in mind the fact that the fish is not feeding at this time, this suggests a synthesis of cholesterol by the herring of about 40 mg. in 6 weeks. The possibility that there had been some mobilisation of sterol from the heads of the fish was not investigated at the time, but has been determined since on heads of fish taken in September 1932. The heads of 8 mature and 8 spent 5-year old fish, for the collection of which we wish to thank Mr W. C. Smith, were saponified in the usual manner and the unsaponifiable fraction was prepared, and the amount of cholesterol present determined. The average figures for the heads of these fish were as follows:

	Wt. of unsap. fraction mg.	Wt. of cholesterol mg.
Mature fish	63	47·1 per head
Spent fish	54·5	41·2

The differences between the figures for the mature and spent fish are insignificant, and hence we incline to the view that the fish is synthesising sterol during the later stages at least of the maturation period.

Summary.

1. A quantitative study has been made of the fatty acids and cholesterol in the different tissues of the Manx herring from the time when the gonads are empty, through the stages of complete maturation to the spawning of the fish about 12 weeks later. Age, weight, length and sex have been considered.

2. The percentages of fatty acids in the liver and "mesentery" increase for 3 weeks to a maximum (15 and 39 % respectively) and then fall to about 1 % by the time of spawning. The maximum in the muscles, 17·4 %, occurs rather later (about 5 weeks) and then progressively falls, approaching the initial value. Meanwhile the rapidly growing ovaries and testes show varying percentages throughout.

3. The percentage of cholesterol in the gonads, however, continuously rises to a maximum with maturation. That present in the liver and "mesenteric" fat disappears at a far slower rate than do the fatty acids of these tissues, while the percentage in the muscle remains approximately constant throughout.

4. Because, with tissues changing so rapidly in weight, the percentage values give no idea of the absolute changes involved, an attempt has been made to interpret the data along these lines. The probability of the assumptions necessary for this purpose is discussed. The balance-sheet so constructed shows that the loss of fatty acid from the time when the maximum values obtain to the time of maturation are: liver 0·25 g., "mesentery" 2·65 g., muscle 3·76 g. Meanwhile the gonads have gained 0·98 g. The significance of these various changes is discussed.

5. It shows further that, in the last stages of maturation at least, the fish is synthesising cholesterol.

6. The degree of unsaturation of the fatty acids of the ovaries and testes rises steadily to a maximum before the fish spawns, when the iodine value has risen from 137 to 200. The iodine values of the acids of the other tissues have however remained constant, the values being for liver 128, mesentery 108, muscle 118. The possible significance of the finding is discussed.

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CCXLII. VITAMIN B_4.

By HAROLD BARNES, JOHN RICHARD PERCIVAL O'BRIEN and VERA READER[1].

From the Department of Biochemistry, Oxford.

(*Received October 29th, 1932.*)

In earlier publications [Reader, 1929; 1930, 1, 2] the evidence has been presented for the conclusion that crude watery or alcoholic extracts of yeast contain two heat- and alkali-labile factors necessary for the nutrition of the rat; in the literature these two factors are referred to as vitamin B_1 (the antineuritic vitamin) and vitamin B_4. A method of assay of vitamin B_4 has been described in detail by Reader [1930, 2] and has since been used with success by Halliday [1932]. As an appendix to the above paper [1930, 2] Reader described a method for preparing vitamin B_4 concentrates free from vitamin B_1, making use of the mercuric sulphate fraction obtained in the Kinnersley and Peters process for vitamin B_1. A stable concentrate (activity 0·6 mg. per rat day dose) was obtained, but further purification seemed impossible without loss of stability; consequently the method was abandoned for one in which the vitamin B_4 was removed from the yeast extracts by adsorption on charcoal at p_H 1·0 [*vide* Peters, 1931].

From these activated charcoals we have now prepared a white crystalline material of high vitamin B_4 activity and of apparently constant chemical composition. Essential steps in this new process are fractionation with sodium phosphotungstate [*cf.* Peters, 1930] and liberation of an actual base by hydrolysis of a base-pentose compound. We hesitate to describe our compound as vitamin B_4 itself, as we have certain indirect evidence as yet unpublished (Kinnersley and Peters), that a preparation of vitamin B_4 of higher activity can be obtained. Our crystals, stored in a desiccator over calcium chloride, have remained stable for 6 months and so form a useful standard for further work on vitamin B_4.

Experimental.

Testing. Throughout this work the adult rat curative method of assay [Reader, 1930, 2] was used to follow the vitamin B_4 activity. As previously mentioned our stock colony has been rigorously inbred in order to make the variation in response between the individual animals as small as possible. At least 5 animals have been used for testing the extracts after each stage in

[1] A preliminary account of this work has previously been published [Barnes, O'Brien and Reader, 1932]. Although the research is still incomplete we have decided to publish a detailed account, as one of us (V. R.) is unable to continue the work.

each preparation. After the phosphotungstate stage not more than 2 in any 20 animals gave results outside a ± 20 % range.

Plate IX shows (A) the condition of an animal when ready for test, and (B) the state of the same animal after 1 week's treatment with the active substance. When in condition (A), the animal is unable to walk without falling (usually to the left side), and cannot control the action of its head sufficiently to drink from the usual vertical bottle. Lack of co-ordination is the most marked symptom, but is frequently accompanied by a noticeable dilation of the capillaries between the digits of the paws.

Purification of active material. The preparation of vitamin B_4-active charcoals from yeast has been described elsewhere [Peters, 1931]. In this paper the technique used in preparing the crystalline material from these charcoals is described. The quantities of materials stated correspond to those required for the charcoals from one batch of 50 kg. of the original yeast. The p_H values were determined by the colorimetric method and are only approximate.

(*a*) *Extraction.* Immediately after the adsorption process, the charcoals were filtered on Büchner funnels, washed with dilute sulphuric acid (p_H 1) and then covered with 50 % ethyl alcohol to which was added sufficient concentrated hydrochloric acid to bring the fluid in contact with the charcoal to p_H 1·0. Four successive extractions, each with 800 cc. of fluid, were carried out by warming on the water-bath to 70° and filtering at the pump whilst hot. The total extraction fluids were mixed, sodium hydroxide was added to bring the p_H to 3·0, and the whole distilled *in vacuo* to remove alcohol. The residue, having a volume of approximately 1500 cc., was allowed to stand in cold store overnight.

(*b*) *Mercuric sulphate treatment.* The extract was again adjusted to p_H 3·0, and 350 cc. of Denigès's mercuric sulphate reagent added. After standing for 1 hour, the fluid was filtered, the precipitate being discarded: excess sulphate was removed from the filtrate by addition of hot saturated solution of baryta, until the p_H was 2·0. After standing another 2 hours, the fluid was filtered, treated with sodium hydroxide to bring it to p_H 4·0, and then with H_2S for 4 hours to remove mercury. After again filtering, the excess H_2S was removed *in vacuo*, the temperature not being allowed to exceed 30°, to prevent appreciable reduction of volume. The fluid, about 2000 cc., was allowed to stand in cold store overnight.

This treatment removes a certain amount of material without diminishing the activity of the extract. Such a result is in contrast with earlier experiments [*vide* Reader, 1930, 2] in which the active substance itself was precipitated by the mercuric sulphate reagent. There can be little doubt that the adsorption on charcoal, introduced into the present technique, is responsible for this altered behaviour.

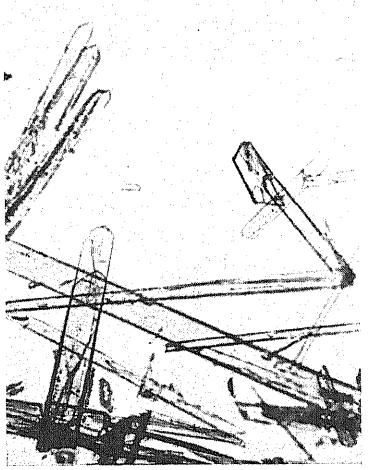
(*c*) *Fractional precipitation with sodium phosphotungstate.* Several preliminary experiments were conducted, in which the merits of the 1:18 and the 1:24 phosphotungstates for fractional precipitation of the vitamin were



(A) Animal suffering from deficiency of vitamin B_4.



(B) Same animal after one week's treatment with active material (0·01 mg. daily).



(C) Crystals with vitamin B_4 activity. ×100.

compared. A typical result is shown in Table I. To each of two flasks, each containing 20 cc. of the fluid (50 rat doses), 1 cc. of the appropriate phosphotungstate was added, and the p_H gradually changed from 6 to 1, precipitates being removed at the points indicated in Table I. Definite zones of precipitation were observed [*cf.* Kinnersley and Peters, 1930].

Table I.

	p_H at which precipitate removed	Approx. no. of vitamin doses recovered
Sodium salt of 1:18 acid	4·0	5
,,	2·5	10
,,	1·0	10
Sodium salt of 1:24 acid	4·0	0
,,	2·5	10
,,	1·0	40

From such results as are shown in Table I it was concluded that a more specific precipitation occurred with the 1:24 salt and that the maximum point for the vitamin was between p_H 2·5 and 1·0. Hence it was decided to use the 1:24 salt (prepared by the method of Wu [1920]) for all further work. Immediately before use 10 g. of the acid were dissolved in 50 cc. of distilled water, 20 % sodium hydroxide was added to bring the p_H to 6·0, and the whole made up to 100 cc.

The fluid from the mercury stage (*b*) was then brought up to p_H 6·0 and an excess of the 10 % phosphotungstate solution (about 50 cc.) added. 5 % sulphuric acid was gradually added to bring the whole to p_H 3·0. It was then allowed to stand in the cold store overnight. Next morning it was filtered and the precipitate discarded. 5 % sulphuric acid was again added to bring the filtrate to p_H 1·0 and the mixture allowed to stand in the cold store overnight. After filtration at the pump, the precipitate, which was straw-coloured, contained the activity.

(*d*) *Recrystallisation of the active phosphotungstate.* The above precipitate was washed through the filter-paper with the smallest possible quantity of hot 50 % ethyl alcohol (about 100 cc.) and allowed to stand in a cool place for 24 hours. Any precipitate which was not soluble in the hot alcohol was discarded.

(*e*) *Removal of phosphotungstic acid.* Next day the yellow deposit (usually, in the main, crystalline) was filtered from the alcohol, dissolved in 50 % acetone, and cold saturated baryta added until the solution turned phenolphthalein pink. It was then shaken well for 5 minutes and filtered as rapidly as possible. A few drops of dilute sulphuric acid were added to the filtrate immediately to remove any excess barium and to make the solution acid again (p_H 3). This filtrate, volume about 250 cc., and containing 30–40 % acetone, should contain 10–15 rat day doses of vitamin B_4 per cc. Test doses were always given to the animals before proceeding further. The acetone

present in 1/10 cc. of the solution was enjoyed by the animals and did not appear to do them any harm. The small deposit of barium sulphate was removed by decantation or filtration immediately before use. In all 19 lots, each representing 50 kg. of yeast were brought to this stage. All were free from vitamin B_1 and from the streptothrix factor [Reader, 1928] and have remained stable for many months.

(*f*) *Hydrolysis.* Acetone was removed *in vacuo* and the fluid concentrated to 50 cc. 5 cc. of concentrated hydrochloric acid were added and the whole was heated on the water-bath for 1–2 hours, or until a positive orcinol reaction for pentose was obtained. The time taken for this hydrolysis varied from 1 to 3 hours in the 17 lots tried, but the average time was 1 hour. It is useless to proceed to the next stage unless a positive orcinol reaction is obtained.

(*g*) *Crystallisation.* The 50 cc. fluid from the above were concentrated to 10 cc. on the water-bath and 20 cc. of acetone added. If any precipitate formed it was removed by filtration and discarded. (Last traces of barium sulphate and chloride were thus removed.) Then 70 cc. of ether were cautiously added and, after gentle stirring, the mixture was allowed to stand on ice for 12 hours or until crystals appeared. On two occasions the crystals appeared within 2 minutes from the time of addition of the ether. The product was recrystallised from a 1 part water–5 parts acetone–50 parts ether mixture. A photomicrograph of the final product is shown in Plate IX (C).

Results.

During the last year about a ton of yeast has been worked through the stages described above. The results from 12 successive lots, each of 50 kg., are recorded in Table II. The numbers represent the total number of rat day doses (curative) in the extracts.

Table II.

Lot	Extract from charcoal	After mercury	After phospho-tungstic	After hydrolysis	Remarks
1	5000	5500	4000	4000	No pentose and no crystals
2	6000	5500	3000	Nil	—
3	6000	6000	5000	4000	30 mg. crystals
4	4500	4000	4000	3500	28 mg. crystals
5	5000	4500	4500	3000	15 mg. crystals
6	6000	5500	5500	3000	No pentose and no crystals
7	5500	5000	5000	4000	20 mg. crystals
8	4500	4200	4000	2500	10 mg. crystals
9	4800	4500	4500	Nil	—
10	6000	6000	5500	5000	5 mg. crystals
11	6000	5000	5500	4500	Nil
12	6500	6000	5000	1000	Nil

It will be seen that the method worked fairly constantly without great loss of activity up to the end of section (*e*), *i.e.* liberation from phosphotungstic acid: but that for the hydrolysis and crystallisation stages the conditions are not yet well defined.

The improvement in purity as the preparation proceeds is shown in Table III. Lots (3), (4) and (5) from Table II are quoted as illustrations.

Table III.

Stage	Activity (organic solid weight required per unit dose (± 20 % variation)). mg. Lot 3	Lot 4	Lot 5
Extract from charcoal	10	9	10
After mercury	5	4	6
After phosphotungstic	0·5–0·2	0·5–0·3	1·5–0·5
Crystalline material	0·010	0·012	0·010

Analysis. Samples of crystals from lots 3 and 5 have been analysed by Dr Ing. Schoeller of Berlin. His findings are:

	C %	H %	N %	Cl %
Lot 3	35·5	3·5	38·03	21·6
Lot 5	34·8	3·4	37·70	22·2

The above figures were obtained after drying the substance at 80° in high vacuum over P_2O_5. The water content was 5·2 % and 5·4 % for the two samples respectively. The empirical formula is probably $C_4H_4N_4.HCl, \frac{1}{2}H_2O$.

Properties. Hydrochloride of a base: sublimes at 220°: very soluble in water: insoluble in absolute alcohol, ether or acetone: forms a crystalline picrate which melts at 278°: is adsorbed on charcoal at p_H 2·0: precipitated by sodium phosphotungstate (1:24) from a 1 in 500 solution at p_H 4·8: gives negative results for the following colour reactions: Pauly, Fehling, Millon, α-naphthol, and Tollens.

Stability to acid. The final product, a base forming salts with acids, is stable to acid treatment, even boiling with 20 % HCl for an hour. At first sight this does not harmonise with earlier results of Reader [1929], in which it was shown that after boiling a watery extract of yeast or marmite for an hour with 5 % HCl, a 50 % destruction of the vitamin had occurred. Again after liberation from phosphotungstic acid, the activity of the solution remains stable so long as acetone or alcohol is present but diminishes on storage, even at 0° in an aqueous solution. It would appear therefore that the stability of the product towards acid is variable and is influenced by the degree of purification achieved.

Stability to alkali. At all stages in the preparation the vitamin B_4 activity is lost on boiling with alkali. The final crystalline material is inactivated by heating on the water-bath with 10 % sodium hydroxide for 10 minutes. So far none of the crystalline base has ever been recovered after this treatment, but this may be due to the small amounts of material handled.

SUMMARY.

An account is given of an attempt to isolate vitamin B_4 from yeast. An unknown base, with a high and apparently constant vitamin content, has been isolated in the form of colourless crystals. Essential steps in its preparation are fractional precipitation with phosphotungstate, and removal of a pentose group from the molecule by acid-hydrolysis. Analyses suggest the formula $C_4H_4N_4.HCl, \frac{1}{2}H_2O$. Further work is required before it can be claimed that the product is identical with the vitamin.

In conclusion we wish to thank Prof. R. A. Peters for his advice and encouragement throughout the research, and to acknowledge gratefully a personal grant from the Medical Research Council to one of us (V. R.); also to thank Miss M. Kempson for her assistance and care with the animals.

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CCXLIII. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

XI. THE METABOLISM OF METHIONINE AND RELATED SULPHIDES.

By NORMAN WINGATE PIRIE.

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(*Received October 30th, 1932.*)

It has long been known that only a fraction of the sulphur in many proteins is present in the form of cystine [Johnson, 1911; Harris, 1923] and it has been known for several years that a part of the remaining sulphur is in the form of methionine. I have already shown that methionine is the principal sulphur-containing amino-acid in caseinogen; this is probably also true in the case of egg-white, for I have isolated, by the same method, 2·5 % of methionine from it. Baernstein [1932], having assumed that proteins contain no methoxyl compounds and no methylthiol compound other than methionine, has published values for the methionine content of a large number of proteins. The validity of his assumptions is doubtful but the values obtained, when taken in conjunction with the cystine contents, enable him to account for approximately 100 % of the sulphur in the proteins used. In most cases the greater part of the sulphur is found to be in the form of methionine. It appears therefore that methionine is generally the principal sulphur-containing constituent of proteins other than scleroproteins and is quantitatively the most important sulphur compound in an ordinary diet. Jackson and Block [1931] and Weichselbaum, Weichselbaum and Stewart [1932] have shown that methionine will supplement a cystine-low diet, but the problem of preparing a diet that is free from either cystine or methionine is still unsolved.

Mueller [1924] showed that, in man, methionine was readily oxidised to sulphate; his experiments were necessarily short and one cannot determine from them the completeness of oxidation of the amino-acid. The present work is an extension of that of Mueller. Dogs have been used and methionine has been compared with cystine on the one hand and with some other sulphides, namely *S*-benzylcysteine, *S*-ethylcysteine and *S*-methylcysteine, on the other.

Methionine is readily oxidised by the dog. The results of three experiments are shown in the figure and are analysed in the table on p. 2042.

The obvious similarity between the metabolic behaviours of methionine and cystine is interesting in view of their widely different chemical structures.

S-Ethylcysteine [Clarke and Inouye, 1931], although closely related chemically to methionine is not oxidised to any appreciable extent by the dog. It is

Substance	Dog	mg. S given	% excreted as sulphate			% excreted as neutral S			% of dose accounted for
			1st day	2nd day	Total	1st day	2nd day	Total	
Methionine	M	320	46	13	59	10	10	20	79
„	P	320	47	19	66	11	9	20	86
„	P	320	58	14	72	9	0	9	81
Cystine	M	320	49	11	60	6	4	10	70
„	P	320	60	11	71	10	3	13	84

These results may be summarised thus.

	% excreted as sulphate in 2 days	% excreted as neutral sulphur in 2 days
Methionine	66	16
Cystine	66	11
Cystine and cysteine [Hele and Pirie, 1931]	70	4

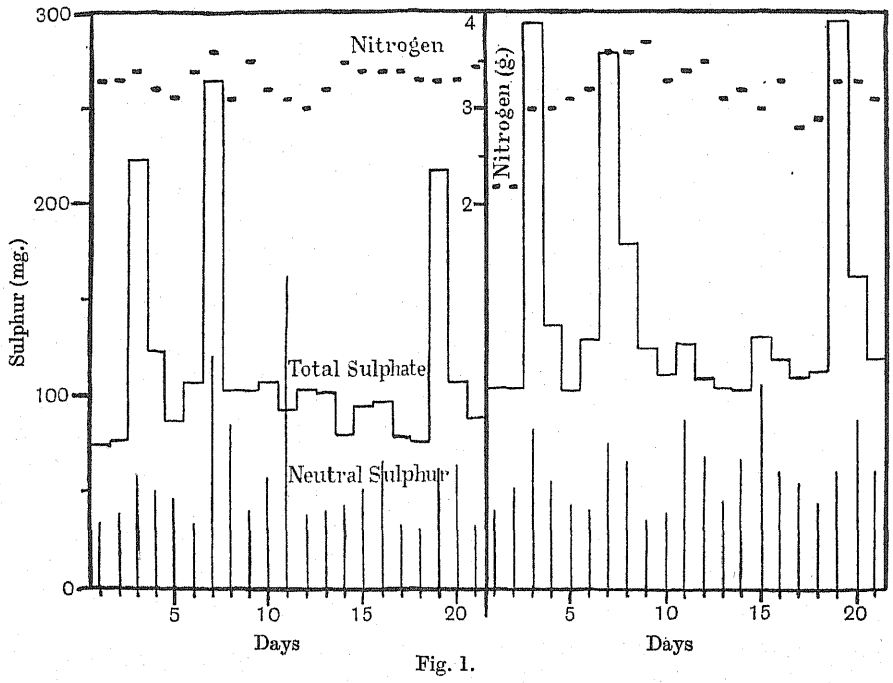


Fig. 1.

MAUD.

Day 3. 1·2 g. cystine orally (320 mg. S)
„ 7. 1·46 g. cysteine HCl subcut. (300 mg. S)
„ 11. 2·11 g. *S*-benzylcysteine orally (320 mg. S)
„ 15. 0·84 g. *S*-ethylcysteine orally (179 mg. S)
„ 19. 1·49 g. methionine orally (320 mg. S)

PATSY.

Day 3. 1·2 g. cysteine orally (320 mg. S)
„ 7. 1·49 g. methionine orally (320 mg. S)
„ 11. 1·49 g. *S*-ethylcysteine orally (320 mg. S)
„ 15. 1·09 g. *S*-benzylcysteine orally (164 mg. S)
„ 19. 1·49 g. methionine orally (320 mg. S)

slightly toxic; both dogs were somewhat lethargic for an hour or two after the dose while one of them (Patsy) vomited a little 11 hours after the larger dose (1·49 g.). The metabolic upset must have been slight since there was no marked effect on the excretion of nitrogen.

No very definite conclusions could be drawn concerning the oxidation of *S*-methylcysteine[1]. Both dogs vomited when given 0·8 g. orally while Maud was made seriously ill by 0·5 g. given subcutaneously, in this case there was also a trace of blood in the urine. Smaller doses than these would contain so little sulphur that it would be difficult to tell whether much had been oxidised or not. There was a definite rise in the total sulphate excretion on both the occasions when the substance was fed, corresponding to 25 % of the dose in the case of Maud and 40 % in the case of Patsy. In each case the urine was contaminated and the effect of the general disturbance on the sulphur metabolism is unknown. It seems to be fairly certain however that *S*-methylcysteine is more readily oxidised than *S*-ethylcysteine but much less readily oxidised than its homologue methionine. It would be interesting to study the behaviour of *homo*methionine and *S*-ethyl*homo*cysteine, *i.e.*

$CH_3SCH_2CH_2CH_2CH(NH_2)COOH$ and $CH_3CH_2SCH_2CH_2CH(NH_2)COOH$.

Differences such as those already mentioned in the metabolism of closely related substances emphasise the need for extreme caution in drawing conclusions, such as those of Sherwin, Shiple and Rose [1927], from the metabolic behaviour of derivatives of cystine or cysteine of unrelated character.

S-Benzylcysteine was prepared according to Suter [1895]. When given by mouth (1·09 g.) it was oxidised to a slight and probably not significant extent by one dog while the other dog did not oxidise a larger dose (2·11 g.) at all. These results contrast with those of Sherwin, Shiple and Rose [1927] who found that the rabbit could oxidise 40 % of the sulphur to sulphate after oral administration of *S*-benzylcysteine. Differences of this sort may easily be due, as Lewis [1924] has pointed out to differences in the intestinal flora of different species. It is noticeable that with *S*-benzylcysteine, as with *S*-ethylcysteine, only about a third of the extra sulphur fed is accounted for in the total sulphur value of the urine. This low recovery is not due to the deficiencies of the method of estimation used [Pirie, 1932, and Appendix to this paper] for control experiments show that 80–90 % of the sulphur in *S*-benzylcysteine or *S*-ethylcysteine added to urine, is converted into sulphate by this method of incineration. There was no change in the excretion of ethereal sulphate after feeding *S*-benzylcysteine. It was noticed that both *S*-benzylcysteine and *S*-ethylcysteine caused a transient diuresis, the volume of urine passed in the course of 24 hours was not increased but in all cases more than usual was passed during the first 12 hours.

Feeding experiments have also been carried out with a number of

[1] The preparation of *S*-methylcysteine has not apparently been described before. It was made by the standard technique with dimethyl sulphate and cysteine hydrochloride. The preparation will be described shortly.

sulphoxides; these will be described fully in a later paper. Preliminary experiments show that methionine sulphoxide is as readily oxidised as methionine whereas the sulphoxides of *S*-ethyl- and *S*-benzylcysteine are completely resistant to oxidation by the dog.

Experimental.

Two bitches, Maud and Patsy, were used. The former has not been used for metabolic work before while the latter is a veteran. The course of the experiments was exactly the same as in the earlier papers of this series [Hele, 1924] and the dogs received the following diets:

	Lean meat g.	Sugar g.	Margarine g.	Milk cc.	Water cc.	Agar g.
Maud (7·3 kg.)	90	60	20	160	100	5
Patsy (7·7 kg.)	70	50	20	160	100	5

There was no alteration in the dogs' weights during the experiments. Agar has recently been added to our standard dog diet since some unpublished work, carried out in collaboration with Hele, has shown that it increases the absorption of some inorganic constituents of the diet.

Total sulphate estimations were carried out by Folin's [1905] method and total sulphur by the method described in the Appendix to this paper. Inorganic sulphate estimations, by the benzidine method, were only made after feeding benzylcysteine; they showed that there had been no change in the excretion of ethereal sulphate.

Summary.

Methionine is oxidised by the dog to the same extent as cystine.

S-ethylcysteine and *S*-benzylcysteine are not appreciably oxidised.

S-methylcysteine is oxidised to some extent but is too toxic to allow an accurate estimate of its oxidation to be made.

I am grateful to Sir F. G. Hopkins for his interest in this work. The expenses of the research were largely covered by a grant to Dr Hele from the Royal Society.

APPENDIX.

Determination of total sulphur in dog's urine.

The estimation of total sulphur in urine is much the most laborious part of the work entailed in studying sulphur metabolism. We have hitherto used Folin's [1905] method; this involves fusion with sodium peroxide and it is the fusion that is most troublesome. The subsequent precipitation of barium sulphate and weighing is, we think, unavoidable with dog urine. Those methods which employ a titration (*e.g.* using benzidine [Fiske, 1921] or barium chromate [Morgulis and Hemphill, 1932]) involve the preliminary removal of phosphate from the urine. I have found that the published methods of doing this, although no doubt admirable when used with human urine, remove part of the neutral sulphur from dog's urine.

The incineration technique now adopted is based on that of Lematte, Boinot and Kahane [1927].

The oxidising agent consists of 3 volumes of concentrated nitric acid and 1 volume of 60 % perchloric acid, 3 volumes of this mixture are then mixed with 1 volume of the same mixture saturated with copper nitrate. The reagent is perfectly stable.

5 cc. of filtered urine and 5 cc. of the acid mixture are heated in a 6 × 1 in. monax test-tube which is two-thirds immersed in an air-bath at about 200°. The temperature is allowed to rise slowly and after 2–3 hours the contents of the tube are dry. It is removed from the bath, allowed to cool for a minute, and 1 cc. of the acid mixture added. After a further 2–3 hours in the bath at a temperature up to 280° the contents will be black or a dirty green, they should not still be blue. The tube is now held in crucible tongs while being heated all over for a few seconds in a large, but not very hot, Bunsen flame. If the heating in the air-bath has been adequate there will only be a few flashes, due to residual perchlorate, in the upper parts of the tube. When cool 2 cc. of *N* hydrochloric acid are added and the tube is replaced in the air-bath while latter is cooling. Boiling water is added till the tube is two-thirds full followed by 5 cc. of the 5 % barium chloride solution. The barium sulphate may be filtered off and weighed in the usual way after 2–4 hours. As a rule the quantity of barium sulphate weighed is of the order of 20 mg., the crucibles must therefore be packed carefully. I have found no difficulty in getting duplicates consistently agreeing to within 0·3 mg.; since the incineration and precipitation are carried out in the same vessel loss can only occur during the filtration. The same Gooch crucible can safely be used 4–5 times without re-packing with these small amounts of sulphate.

The air-bath used consists of a heavy iron saucepan with an inch of sand in it, an unglazed earthenware plate which is 1 inch less in diameter than the pot is supported on legs over the sand. The tubes and a thermometer pass through holes in an asbestos sheet cover and rest on the plate. The whole is heated by a large burner. Twelve tubes can conveniently be heated simultaneously.

This method has been checked on a number of different dog urines against my own estimations and those of Hele with the peroxide method. The differences were within the experimental error.

When used with materials having a low salt content, amino-acid fractions from a protein for example, the incineration can be carried through in an hour and, if very accurate results are not required, the estimation may be finished by the benzidine method.

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CCXLIV. THE COLORIMETRIC DETERMINATION OF PHOSPHORUS IN THE PRESENCE OF INTERFERING SUBSTANCES.

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Recently, the present authors [Davies and Davies, 1931] described a method for the elementary analysis of phosphorus in pure organic compounds, by a modification of the Briggs-Bell-Doisy method. It was observed during the course of the above investigation that it was necessary to standardise the conditions exactly, in order to ensure accuracy. At the same time, one of us was working with Dr J. H. Quastel, in the Biochemical Laboratory of the Cardiff City Mental Hospital, upon the presence of phosphatases in brain tissue. It was then noted that sufficiently large quantities of potassium pyrophosphate prevented the formation of the characteristic blue acid, due to the reduction of the yellow phosphomolybdic acid, resulting from the interaction between molybdic and orthophosphoric acids.

Fiske and Subbarow [1925], Vásárhelyi [1929], and King [1932] have shown that 1:2:4-aminonaphtholsulphonic acid, used in the place of quinol for the reduction of the yellow phosphomolybdic acid, diminishes the effect of many substances, such as sodium chloride, sodium nitrate and ammonium sulphate, all of which hinder the formation of the blue colour. Denis and Meysenbug [1922] found that in certain cases the original Bell and Doisy method gave anomalous results with both citrated and oxalated plasmas, and recommended that it was desirable to restrict the amount of anticoagulant when estimating the inorganic phosphate of blood. Whilst this paper was being written, Tschopp and Tschopp [1932] reported that a quantitative estimation of the orthophosphate ion by their modification of the colorimetric method was only possible when there were less than 30 mg. of ammonium oxalate, or 50 mg. of sodium citrate or 50 mg. of tartaric acid per 0·04 mg. of phosphorus present. They also discussed the effects of ferrous, ferric, cuprous and manganous ions and also of silicates and arsenates.

In the present work it has been found that a number of organic acids and their salts, which may often occur together with phosphorus-containing compounds in biological material, or may be added during biochemical manipulations, can, if present in sufficiently large quantities, entirely prevent the formation of the blue colour. Hence it has been felt necessary to investigate

the maximum quantity of the interfering substance with which it is possible to estimate orthophosphate satisfactorily by the colorimetric method. When the amount of interfering substance is sufficiently large to affect the procedure, we have sought to modify our method to enable us to carry out the estimation accurately.

The effect of sodium and potassium pyrophosphate.

Table I illustrates the effect of increasing quantities of potassium pyrophosphate upon the estimation of 0·08 mg. of phosphorus present as potassium dihydrogen phosphate.

Table I. *The effect of potassium pyrophosphate.*

Tube No.	Mg. potassium pyrophosphate	Mg. phosphorus recovered	Percentage recovered	After 12 hours. Mg. phosphorus recovered
1	0	0·08	100	—
2	12·5	0·083	104	—
3	25	0·086	107·5	—
4	30	0·093	116	—
5	35	0·083	104	—
6	37·5	0·076	95	—
7	40	0·071	89	—
8	45	0·048	60	—
9	50	0·020	25	0·25
10	62·5	0·0	0	0·16
11	75	0·0	0	0·06
12	87·5	0·0	0	0·0
13	100	0·0	0	0·0

To each tube were added 1 cc. of potassium dihydrogen phosphate solution containing 0·08 mg. of phosphorus, increasing quantities of potassium pyrophosphate, 2 cc. of 5·5 *N* sulphuric acid, 2 cc. of 5 % ammonium molybdate, distilled water to about 12 cc. and 1 cc. of reducing agent (15 % sodium sulphite and 0·5 % quinol). The final volume was made up to 15 cc. with water and the contents of the tubes were allowed to stand for 30 minutes. At the end of this period the colour intensity in each tube was matched against an appropriate standard in a Klett top-reader colorimeter. It was so arranged that the colours of the standard and test solutions were almost identical.

Until a concentration of 2·5 mg. (No. 6) of potassium pyrophosphate per 1 cc. of reaction mixture was reached a more than theoretical recovery of orthophosphate was obtained. This was probably due to hydrolysis of the pyrophosphate in the acid solution producing orthophosphate, and perhaps to some extent slight contamination of our pyrophosphate sample. It should be noted, for example, in No. 4 in which the maximum increase in recovery of phosphorus was obtained, that this represented less than 0·3 % of the phosphorus content of the pyrophosphate present. Above concentrations of 2·5 mg. per cc. of reaction mixture the colour became less intense and finally disappeared at concentrations above 4 mg. The last column of Table I shows the effect of allowing the tubes to stand for 12 hours. This series gives further information concerning the nature of the hydrolysis of the pyrophosphate (see p. 2053).

The graphs, Fig. 1, show that the amount of sodium pyrophosphate necessary just to prevent the formation of the blue colour is proportional to the concentration of the ammonium molybdate reagent. The three series of experiments here figured only differ from those given in Table I in the quantities of ammonium molybdate added. Thus, using 1, 2 or 3 cc. of molybdate complete absence of colour commences when 2, 4 or 6 mg., respectively, of sodium pyrophosphate per cc. of the reaction mixture are added. These results indicate that the mechanism of the inhibition is one of complex salt formation between the alkali pyrophosphate and the ammonium molybdate. At con-

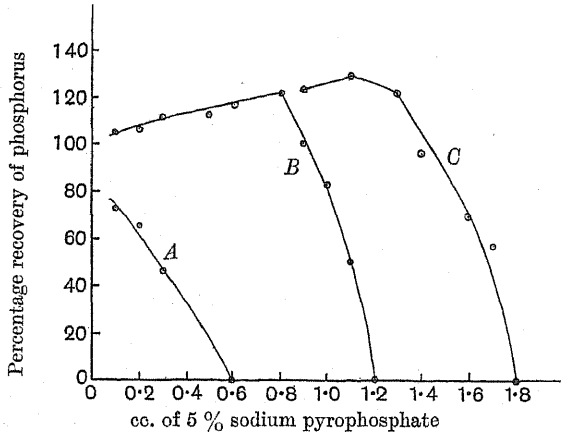


Fig. 1. Effect of variation of the amount of ammonium molybdate on the recovery of orthophosphate (0·08 mg. of phosphorus) in the presence of increasing amounts of sodium pyrophosphate. *A*, 1 cc., *B*, 2 cc., *C*, 3 cc. of 5 % ammonium molybdate.

centrations where complete absence of blue colour occurs, there is sufficient pyrophosphate present to use up the whole of the molybdate, so that no phosphomolybdate can be formed for ultimate reduction to the blue complex. The addition of more molybdate results in the reappearance of the blue colour.

Table II shows the effect of two different reducing agents and of increasing the hydrogen ion concentration on the recovery of orthophosphate from solutions containing increasing amounts of sodium pyrophosphate.

The tubes were made up as described for Table I, except for the acidities, which are given in the table, and the reducing agents, which are given below.

Reducing agent A. 1 cc. of quinol reagent. The colours were compared with the standards after 30 minutes with this reagent.

Reducing agent B. 0·5 cc. of 1:2:4-aminonaphtholsulphonic acid reagent (0·5 g. of the acid, 30 g. of sodium bisulphite and 6 g. of sodium sulphite made up to 250 cc. [King, 1932]). The colours were compared with the standards according to the directions given at the head of the table.

Under the standard conditions of acidity (2 cc. of 5·5 *N* H_2SO_4) it will be seen that with 1:2:4-aminonaphtholsulphonic acid as reducing agent rather more pyrophosphate was required to cause complete inhibition than with

Table II. *The effect of different reducing agents and of increased acidity.*

		Percentage recovery of phosphorus				
		Reducer A		Reducer B		
		Acidity in cc. 5·5 N H_2SO_4				
Tube No.	Mg. sodium pyrophosphate	2 cc.	4 cc.	2 cc. Read after 5 mins.	2 cc. After 20 mins.	4 cc. After 5 mins.
1	5	106	<50	—	—	<50
2	10	106·5	|	100	—	|
3	15	112	Gradual decrease	—	115	Gradual decrease
4	20	—	with no sharp	73	—	with no sharp
5	25	112·5	break	—	116	break
6	30	117	|	67	—	|
7	35	—	|	—	116	|
8	50	83·5	|	—	114	0
9	55	—	0	—	—	
10	60	0		—	—	
11	65	—		—	96	
12	75	—		—	<50	
13	85	—		0	—	
14	90	—		—	0	

quinol. The difference might possibly be ascribed to the greater sensitivity of the naphthol derivative as a reducing agent. Because of the increased time for which estimations with quinol have to be allowed to stand before comparison the hydrolytic process becomes apparent. This is also shown if the colours developed with reagent B are allowed to stand and then matched.

When the acidity is increased the weakening in the blue colour occurs with much smaller concentrations of pyrophosphate, but the point at which colour completely disappears is almost the same using either of the reducing agents. A similar decrease in the intensity of the blue colour is seen even in the absence of pyrophosphate when the acidity is increased, so that no importance can be attached to the above result.

It is only possible to estimate orthophosphate accurately by the colorimetric method when there are less than 10 mg. of alkali pyrophosphate present per 0·1 g. of ammonium molybdate. Also the reducing agent used should be one with which maximum colour intensity is reached rapidly (*e.g.* 1:2:4-aminonaphtholsulphonic acid), and the colour developed must be compared with the standard within 5 minutes of completing the addition of the reagents to the test solution. It is advisable to maintain the acidity at 2 cc. of 5·5 N sulphuric acid per 15 cc. of reaction mixture.

In the present case it is difficult to suggest the actual combining proportions of the molybdate and the pyrophosphate, because of the lack of accuracy in determining the end-point. For example, from Table II, column 5, tube 13, it is found that 85 mg. of sodium pyrophosphate combine with 100 mg. of ammonium molybdate, from which, by calculation, 0·3363 mols. of pyrophosphoric acid combine with 1 mol. of MoO_3. Compounds of pyrophosphates and tungstic acid have been described by Gibbs [1885] of the general formula

$$9M_4P_2O_7,\ 22WO_3,\ xH_2O.$$

From the above calculation the following relation holds:

$$0{\cdot}3363H_4P_2O_7 : MoO_3 :: 7{\cdot}4H_4P_2O_7 : 22MoO_3.$$

In respect, however, to the formulae of complex compounds of these types, it should be remarked that the degree of acidity of the reacting solutions has a very marked influence. Wu [1920] has drawn particular attention to this point.

Sodium glycerophosphate.

The effects of increasing quantities of sodium glycerophosphate on the production of blue colour were very similar to those described above with alkali pyrophosphate. Table III shows the interfering and hydrolytic effects in the acid solution. It was difficult to determine the exact point at which colour just disappeared because the colours were visibly increasing in intensity whilst they were being compared.

Table III.

Tube No.	cc. sodium glycerophosphate	Percentage of phosphorus recovered
1	0	100
2	0·5	118
3	0·75	118
4	1·0	118
5	1·1	95
6	1·2	Gradual
7	1·5	decrease
8	1·9	0
9	2·2	No recovery after an interval of 10 mins.

Each tube contained 1 cc. of potassium dihydrogen phosphate solution containing 0·08 mg. of phosphorus; increasing amounts of sodium glycerophosphate solution (12·24 g. of $Na_2C_3H_7O_6P, 5H_2O$ dissolved in water and made up to 100 cc., which then contained 0·04 mol. of the salt); 2 cc. of ammonium molybdate solution (6·1815 g. dissolved in water and made up to 100 cc., which then contained 0·005 mol. of the salt); 2 cc. of 5·5 *N* sulphuric acid; 0·5 cc. of 1:2:4-aminonaphtholsulphonic acid; and water to 15 cc. Colours were compared after 5 minutes. When allowed to stand for 12 hours, the contents of all the tubes became deeply coloured.

On the assumption that at the point at which the colour first disappears there are present exactly equivalent amounts of ammonium molybdate and sodium glycerophosphate to form a complex salt, it is possible to suggest the combining proportions of the two substances. By experiment, 1·9 cc. of sodium glycerophosphate solution combine with 2 cc. of ammonium molybdate solution from which, by calculation, 1·08 mols. of glycerophosphoric acid combine with 1 mol. of MoO_3. A series of experiments was carried out using a much smaller concentration of acid (0·5 cc. of 5·5 *N* H_2SO_4 per 15 cc. of total mixture) and showed that the combining proportions were not materially affected.

In a recent paper, Fleury [1932] described a physical and a chemical method for determining complex formation between molybdic acid and alkali glycerophosphates in neutral solution. He also isolated crystalline complex salts from neutral solution of the following composition: $M_2C_3H_7O_6P$, $2{\cdot}5MoO_3$, xH_2O.

It is possible to determine accurately 0·08 mg. of phosphorus present as alkali orthophosphate by the colorimetric method in the presence of less than 20 mg. of sodium glycerophosphate per 0·1236 g. of ammonium molybdate, provided the conditions of the estimation are those described for the accurate determination of phosphate in the presence of alkali pyrophosphate.

The effect of certain organic acids.

Table IV gives a list of certain organic acids which, in sufficient quantity, inhibit the formation of the blue colour due to the reduction of phosphomolybdic acid. In these cases hydrolysis with production of increasing quantities of orthophosphoric acid cannot take place. The point at which colour first disappears when adding increasing amounts of the inhibitor is not so obscure as in the previous cases. Moreover, the range over which accurate determinations of orthophosphate in the presence of these substances can be made is greater than the range in the presence of pyrophosphoric or glycerophosphoric acids.

Table IV.

Interfering acid	Maximum amount in mg. of the interfering acid with which 100 % recovery of phosphorus is possible	g.-mol. of interfering acid added when complete absence of colour is first observed	Combining proportions
Citric acid	30	$3{\cdot}6 \times 10^{-4}$	$0{\cdot}5C_6H_8O_7$, MoO_3
Oxalic acid	50	8·0 ,,	$C_2H_2O_4$, MoO_3
Pyruvic acid	50	10·0 ,,	$1{\cdot}5C_3H_4O_3$, MoO_3
Tartaric acid	90	10·4 ,,	$1{\cdot}5C_4H_6O_6$, MoO_3
Malic acid	120	21·0 ,,	$3C_4H_6O_5$, MoO_3
Lactic acid	120	29·0 ,,	$4C_3H_6O_3$, MoO_3
Glycollic acid	250	64·0 ,,	$9C_2H_4O_3$, MoO_3

The corresponding quantity of ammonium molybdate present was $1{\cdot}0 \times 10^{-4}$ mol., whilst there were 0·08 mg. of phosphorus, and 2 cc. of the standard sulphuric acid. Aminonaphtholsulphonic acid was the reducing agent, and the total volume was finally 15 cc. All the solutions of the interfering substances were exactly neutralised with alkali before use.

A change of the reducing agent to quinol makes no alteration in the figures recorded in Table IV for citric acid. Similarly, with a change of the acidity to 0·5 cc. of the standard acid there is little difference in the figures.

In all cases the colours produced became darker on keeping. Even tubes which originally contained a slight excess of interfering acid became pale blue. Probably the organic acid-molybdate complex is reduced on preservation.

Many methods, both chemical and physico-chemical, have been suggested

for determining complex formation with molybdates. The actual isolation of the complexes is usually very difficult, since they are often non-crystallisable syrups. In general, the results of the various methods appear to be rather conflicting. Darmois [1923] gives a bibliography of previous work on the investigation of mixtures of molybdic acid and other substances. Further work in this direction has been carried out by Fleury [1932]. The experiments reported in the present paper furnish a new method for determining complex formation in acid solution between molybdates and other substances, and give the combining proportions of the two substances. An alkali orthophosphate and a reducing agent are used as indicator of complex formation, and, if the latter takes place, of the point at which all the molybdate is converted into the complex compound. A substance which interferes with the colour development in the manner described herein is probably one which combines with the molybdenum compound. When the interfering substance is in insufficient quantity a blue coloration, which disappears with an excess of the substance, develops. In Table IV combining proportions of molybdenum trioxide and organic acids obtained by this method are tabulated.

The following substances even in large amounts do not interfere with the colour development: formic, acetic, succinic, maleic, fumaric, and malonic acids, alcohol, glycerol, glycine, urea, glucose and fructose. We have not experimented with sufficient compounds to make a generalisation concerning the type of substance which would be likely to undergo complex formation with ammonium molybdate. However, in this connection it should be noted that all the substances given in Table IV contain the carboxyl group, and, in addition, all except oxalic acid contain a hydroxyl or a carbonyl group.

The competitive effects of orthophosphate and interfering substances for molybdate.

The effect of increasing the concentration of orthophosphoric acid in the presence of sufficient sodium citrate to prevent colour formation under standard conditions was investigated. Each tube contained 85 mg. of citric acid and increasing amounts of potassium dihydrogen phosphate. Colour was developed in the usual way. The colour intensity increased proportionately but did not at any time indicate theoretical recovery of orthophosphate. Table V gives the result of the experiment.

Table V. *The recovery of orthophosphate in the presence of sodium citrate.*

Tube No.	Orthophosphate added in mg. of phosphorus	Recovery in mg. of phosphorus
1	0·08	0
2	0·16	0·038
3	0·24	0·047
4	0·32	0·110
5	0·40	0·125

The results show the competitive effect of the orthophosphate and of the citrate for the ammonium molybdate. As the proportion of the phosphate to the citrate increases so the amount of phosphomolybdate increases, whilst the amount of the citric acid-molybdic acid complex must decrease. A complete explanation of the results is bound up with the relative stabilities of the two complex compounds. Reference to Table I, last column, shows a similar effect. When the contents of the tubes are kept for 12 hours, an increased amount of orthophosphoric acid is produced by reason of the hydrolysis of the pyrophosphate in acid solution. This is made evident by the appearance of a blue colour in tubes which originally contained sufficient pyrophosphate to inhibit the colour.

Similar competitive effects have been observed between citric acid and malic acid for ammonium molybdate. We are indebted to Dr J. H. Quastel for providing us with the following results.

Molecular rotations ($[M]_{5461} = \frac{10\alpha}{l.c}$, where α = the observed rotation, l = 2 dm. and c = the molar concentration per 1000 cc. of *l*-malic acid, at room temperature) of aqueous mixtures of ammonium molybdate with varying amounts of citric acid and malic acid are given in Table VI. The solution was made up of 10 cc. of 14·2 % ammonium molybdate solution, 1 cc. of glacial acetic acid, and 5 cc. of the mixture of malic and citric acids. The concentrations of malic and citric acids given in Table VI refer to this solution.

Table VI. *Molecular rotations of mixtures of ammonium molybdate, malic and citric acids.*

		Citric acid concentration				
		0	*M*/160	*M*/80	3*M*/160	*M*/40
Malic acid concentration	*M*/160	+ 1096°	1888°	2096°	2400°	2400°
	M/80	1120	1688	1900	2000	2128
	M/40	1190	1550	1770	1894	—
	3*M*/80	1177	1493	1659	—	—
	M/20	1195	1464	—	—	—

From Table VI it is evident that (1) the molecular rotation of the malo-molybdate complex increases with increase of the concentration of the malic acid to a maximum; (2) for a given concentration of malic acid, an increase in the concentration of citric acid in the mixture produces an increased molecular rotation which finally reaches a maximum. This shows the formation of a citro-malo-molybdate complex of higher rotation than the malo-molybdate complex; (3) for a given concentration of citric acid in the mixture, an increase in the concentration of malic acid reduces the molecular rotation.

These results indicate the replacement of malic acid by citric acid and *vice versa* from their complexes with ammonium molybdate. The trend of the reaction is governed by the relative concentrations of the combining substances. There is obviously competition by the two acids for the available valencies of ammonium molybdate.

Modified method of analysis.

In order to estimate orthophosphate in the presence of certain organic acids, Tschopp and Tschopp [1932] recommended that the latter be removed by oxidation with potassium permanganate. We suggest that the easiest method of obviating the difficulty arising from the presence of the interfering substance is to add sufficient ammonium molybdate to combine with the inhibitor and then the standard quantity to react with the orthophosphate. For example, with citric acid (neutralised with alkali) as inhibitor, 30 mg. are the maximum amount which can be allowed for theoretical recovery of orthophosphate, when using 2 cc. of standard ammonium molybdate (0·1236 g. of the salt). If, however, 50–70 mg. of citric acid are known to be present, then theoretical recovery of orthophosphate will be obtained by adding double the quantity of ammonium molybdate. This method requires no extra step in the method of analysis, but assumes a knowledge of the quantity of interfering substance present. The quantities of the inhibitor which interfere are large, and are much greater than those obtained under physiological or even pathological conditions. This means that they can only be present in such large amounts when they have been added during the course of experimental manipulation. Under these circumstances the amount of the interfering substance can be assessed. From the figures given in Table IV, a calculation can then be applied to find the amount of ammonium molybdate required. In spite of this, it is recommended that the amount of inhibiting substance, and consequently that of extra molybdate, should be kept as low as possible.

With those interfering compounds which through hydrolysis yield free orthophosphate in the acid solution, the above modification is of no value. The quantities present must be restricted to the maximum allowed.

Summary.

1. Pyrophosphoric, glycerophosphoric, citric, oxalic, pyruvic, tartaric, malic, lactic and glycollic acids interfere with the quantitative determination of orthophosphate by the colorimetric method.

2. The maximum amounts of the interfering compounds which may be present for theoretical recovery of orthophosphate have been determined.

3. The anomaly is caused by complex formation between ammonium molybdate and the interfering substance. Besides forming complexes with ammonium molybdate, pyrophosphoric and glycerophosphoric acids cause further difficulties because they undergo hydrolysis producing orthophosphoric acid.

4. From a knowledge of the amount of interfering substance which is necessary just to prevent the formation of blue colour (from reduced phosphomolybdic acid), combining proportions of molybdate and interfering substance have been suggested.

5. By increasing the amount of molybdate, within certain limits, it is possible to estimate orthophosphate in the presence of larger amounts of the foreign substance than are given in (2).

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CCXLV. CHOLINE-ESTERASE. AN ENZYME PRESENT IN THE BLOOD-SERUM OF THE HORSE.

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The question of the hydrolysis of acetylcholine by enzymes has been brought into prominence in connection with the investigations on the mechanism of nervous action which Loewi has carried out during the past decade. In his original paper on this subject Loewi [1921], working with isolated frogs' hearts, showed that stimulation of the vagus caused the formation or liberation in the heart of a substance which itself produces the effect previously considered to be due to the action of the nerve. It was recognised by Loewi [1922] that this so-called vagus-substance was a choline derivative, while Loewi and Navratil [1926, 1] later advanced evidence which indicated that it was an ester of choline, possibly acetylcholine, although Witanowski [1925], working in Loewi's laboratory, had previously stated that it was more stable than the latter substance. Not only did Loewi and Navratil find that the physiological activities of the vagus-substance and acetylcholine were indistinguishable, but they discovered that both substances were destroyed by aqueous extracts of frog's heart. The destructive agent present in such extracts was thermolabile and possessed other properties characteristic of enzymes; it was therefore considered to be an esterase. Following this work, Galehr and Plattner [1927, 1, 2] demonstrated that acetylcholine was similarly destroyed by defibrinated blood from various species. They were nevertheless of the opinion that no enzyme was involved but that the destruction was brought about by a process of adsorption catalysis, a view which was elaborated in subsequent papers by Plattner and his colleagues. It is unnecessary, however, to consider the latter work in detail since Engelhart and Loewi [1930] have now conclusively demonstrated the enzymic nature of the destructive agent; results of a similar nature have, moreover, been obtained by Matthes [1930].

That acetylcholine should be destroyed by esterases is not surprising. These enzymes, which are fairly widely distributed in the animal organism, are known to hydrolyse a variety of esters, and there seemed no reason to suppose that acetylcholine would be exceptionally resistant to their action. It has long been known, for example, that the blood-serum from many species contains an enzyme which attacks tributyrin, and it appeared logical to attribute the hydrolysis of acetylcholine by blood or serum to the action of this enzyme, and

to assume, in fact, that all esterases, and possibly lipases, would produce the same effect. Nevertheless, as pointed out by Stedman and Stedman [1931], the possibility that the destruction of acetylcholine was effected by a specific enzyme remained. No information was available on this point, for, in the work outlined above, pharmacological methods were employed to follow the destruction of the drug. Owing to the extraordinary activity of this substance, such methods can be and, in fact, were employed with minute amounts of material, the experiments of the above-mentioned authors being carried out with a fraction of a milligram of the drug. While these experiments served to demonstrate the destruction of acetylcholine, it was clear that they could give no information regarding the specificity or otherwise of the enzyme involved; much larger amounts of material would be required for this purpose. Stedman and Stedman therefore attempted to solve this problem indirectly by utilising an observation, made by Loewi and Navratil [1926, 2], that physostigmine, which potentiates the action of the vagus, does so in virtue of the fact that it inhibits the activity of the enzyme which causes the destruction of both acetylcholine and the vagus-substance. Assuming that physostigmine acted specifically on the latter enzyme, as seemed to be indicated by Matthes's results [1930], it appeared that the problem could be readily solved by examining the action of the alkaloid on an authentic esterase. Stedman and Stedman therefore examined the action on liver-esterase, using both methyl butyrate and tributyrin as substrates, of a group of urethanes which they had previously prepared and which, like physostigmine, could be classed as parasympathetic stimulants. In every case the activity of the esterase was inhibited by minute concentrations of the urethanes, a result which afforded strong evidence for the view that the enzyme which destroys acetylcholine is an esterase such as is present in the liver. This view, moreover, received additional support from the subsequent work by the same authors [1932], in which it was shown that whereas the activity of kidney-phosphatase towards glycerophosphoric acid was uninfluenced by the urethanes in question, that of serum-esterase from the guinea-pig towards tributyrin was inhibited to much the same extent as liver-esterase. Further, pancreatic lipase from the pig, although inhibited by relatively large concentrations of the urethanes when methyl butyrate was the substrate, was unaffected when the substrate was olive oil. These results certainly demonstrated that the inhibitory action of urethanes of the type concerned was specific to the extent that it was directed towards true esterases. Indirectly, they seemed to show that the destruction of acetylcholine by blood-serum and tissue extracts was caused by an esterase similar to that present in the liver and not by one of a specific nature.

Action of esterases and lipases towards acetylcholine.

Despite these results it appeared desirable to extend the work and to examine the activity of the various enzymes towards acetylcholine, using chemical methods of estimation. For the first experiments of this kind we

again selected liver-esterase, since preparations of this enzyme containing relatively little protein can be readily obtained by the methods developed by Willstätter and his co-workers [for details, see Stedman and Stedman, 1931]. When, however, the activity towards acetylcholine of such preparations was examined by the procedure described below, none was found. Even with a concentration of enzyme five times as great as that necessary to follow the hydrolysis of methyl butyrate under otherwise identical conditions (p_H 7·5), no hydrolysis of the acetylcholine beyond that due to the hydroxyl ions could be detected. The liver-esterases from two species, namely the pig and the cat, were tested with identical results. Pancreatic lipase from the pig [for preparation, see Stedman and Stedman, 1932] was similarly found to be inactive.

Method of estimation.

In view of these negative results we next turned our attention to the esterase present in horse-serum, this particular species being chosen partly because it had been employed in some of the pharmacological experiments mentioned above and partly because the serum could be readily obtained in quantity. For the estimation, the method of continuous titration, developed by Willstätter, Kuhn, Lind and Memmen [1927] in connection with liver-esterase, and subsequently used by many workers, was utilised under the following conditions. To 100 cc. of water, warmed to 30°, were added a measured volume of a solution of acetylcholine chloride of known concentration, 5 drops of a solution of bromothymol blue, and sufficient 0·0225 N alkali to bring the p_H to about 7·4. A measured volume, usually 1 cc., of the serum or solution to be tested was then added, the flask placed in the thermostat, and, after again adjusting the p_H of the solution, 0·0225 N sodium hydroxide was run into the flask, drop by drop, as required to maintain the p_H of the solution at the above value. Burette readings were recorded every 5 mins. for a period of 20 mins.

While the above method served to demonstrate the hydrolysis of acetylcholine by horse-serum, the results obtained were not as regular as was desired. Two factors were mainly responsible for this: the buffering action of the serum-proteins and the yellow pigment present in the serum. The latter modified the colour of the indicator to such an extent that it became difficult, if not impossible, to maintain the solution at a definite p_H. It was obvious that some purification of the enzyme would have to be effected if its properties were to be examined, and it thus became desirable to devise an improved method of estimation in order to facilitate the process of purification. Two modifications were therefore made in the above procedure. The first depended upon an observation which we made that butyrylcholine is hydrolysed more rapidly by the serum-enzyme than is acetylcholine. The former ester was therefore employed as substrate, a change which possessed a twofold advantage. Not only was there, for a given amount of enzyme, an increased liberation of

acid, but the correction which it was necessary to apply was smaller owing to the fact that butyrylcholine is much less susceptible than acetylcholine to the hydrolytic action of hydroxyl ions. The latter factor was not, it is true, of great importance when the experiments were carried out at p_H 7·4, for at this acidity the correction is almost negligible with either substrate; its advantage lay in the fact that it rendered possible the second modification, which consisted in carrying out the experiments at p_H not less than 8·6, using *o*-cresolphthalein as indicator. At this acidity the buffering action of the serum-proteins was much diminished, and hence the titrations were considerably sharper. Nevertheless, it cannot be claimed that accurate results were obtained with this modified procedure; it was used because it appeared to be the best available for the purpose of tracing the course of purification of the enzyme. When some success had been achieved in this direction, it was possible to revert to the method first described. It should be mentioned that, in order to avoid the possible inhibitory action of alcohol, aqueous solutions of the sodium salts of the indicators, prepared according to Clark [1925], were used throughout the work.

Choline-esterase activity of horse-serum.

It has been shown above that whereas liver-esterase and pancreatic lipase are without action on acetylcholine, an enzyme which is present in the blood-serum from the horse is able to hydrolyse both this substance and butyrylcholine. Although all the solutions of this enzyme which we have hitherto prepared have also attacked both methyl butyrate and tributyrin, their activities towards the latter substrates have been smaller than towards esters of choline. It is possible that different enzymes are involved. Assuming, however, that this is not the case and that the same enzyme has been responsible for the hydrolysis of these different types of esters, it is clear that the enzyme in horse-serum differs from liver-esterase in a manner analogous to that in which the latter differs from pancreatic lipase. It has been shown, for example, by Willstätter and Memmen [1924] that liver-esterase and pancreatic lipase resemble one another in hydrolysing both simple esters and fats, but differ enormously in the relative rates at which they attack these different types of esters, liver-esterase showing a decided preference for the former and pancreatic lipase for the latter type. Similarly, the horse-serum-enzyme, on the above assumption, resembles liver-esterase in hydrolysing methyl butyrate and tributyrin but differs from it in the fact that it also attacks esters of choline, and does so, moreover, more rapidly than it hydrolyses methyl butyrate. In order to emphasise this difference it is proposed to term the enzyme which hydrolyses esters of choline choline-esterase.

The choline-esterase activity of the serum from individual horses varies considerably. This is illustrated by the following experiments. Using, in each case, 0·1 g. of acetylcholine as substrate the acid liberated in 20 mins. at p_H about 7·5 by the enzyme present in 1 cc. of serum was equivalent, with three different sera, to 4·2, 3·6, and 2·45 cc. respectively of 0·0225N alkali. Two

further specimens were tested at p_H about 8·8 with 0·1 g. of butyrylcholine as substrate; the corresponding figures were 7·2 and 4·65 cc. While, for the reasons explained above, no great accuracy is claimed for these figures, it is evident that wide variations in the choline-esterase content occur.

It is interesting to note that serum, if kept sterile, retains its choline-esterase activity practically unimpaired for months. The procedure we have usually adopted is to saturate the serum with chloroform and then to store it in the refrigerator.

Purification of choline-esterase.

In attempting to purify choline-esterase we have avoided devising a unit of activity for which any permanent meaning could be claimed. We have, however, used two magnitudes in order to give numerical expression to the yield of enzyme obtained and to the degree of purification effected in any operation. These magnitudes may, for convenience, be termed the volume-number and weight-number respectively. The former represents the volume, calculated from a determination of the activity of the preparation, of 0·0225N alkali which would be required to neutralise the acid liberated in 20 mins. by the enzyme obtained from 100 cc. of serum; the latter is the volume required to neutralise the acid similarly liberated by that contained in 1 g. of the dry material. In calculating the values of these numbers the assumption has been made that the activity is proportional to the enzyme content; this will, of course, only hold strictly under uniform conditions of estimation. During the progress of this work these conditions have been modified; for example, in the earlier experiments only 0·1 g. of acetyl- or butyryl-choline was used as substrate, whereas it was later found advantageous to employ 0·25 g. The values of the volume-numbers and weight-numbers obtained in different series of experiments will not therefore be comparable. Within any single series, however, where the conditions of estimation have been maintained as constant as possible, the values of these expressions give at least an approximate indication of the yield of enzyme which has been obtained and of its degree of purity. Typical experiments, carried out during the attempted purification of the enzyme, are briefly described below.

Dialysis. Collodion membranes were employed throughout these experiments, dialysis being effected under pressure in order to avoid great dilution.

100 cc. of serum were dialysed until salt-free. After removing a precipitate of euglobulin, 130 cc. of solution were obtained. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1·3 cc. required 4·8 cc. alkali in 20 mins. Volume-number, 480; volume-number for original serum, 465. No loss of activity occurs on dialysis.

Precipitation with ammonium sulphate. Preliminary experiments in which attempts were made to remove the enzyme from the dialysed solution by adsorbing it on alumina and kaolin having failed, precipitation with ammonium sulphate was examined. The following experiments give the results obtained with increasing concentrations of the salt.

20 g. of ammonium sulphate were dissolved in 100 cc. of serum. After 2 hours the solution was centrifuged and the centrifugate and precipitate dialysed separately. The precipitate, which readily dissolved in a small volume of water, gave 29 cc. of an almost colourless solution. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 0·85 cc. alkali in 20 mins. Volume-number, 25. The centrifugate yielded 117 cc. of solution containing 3·5 g. of solid. Estimation: 1 cc. required 4·1 cc. alkali in 20 mins. Volume-number, 480; weight-number, 117. Volume and weight numbers for serum, 465, 55.

In another similar experiment the precipitate from 200 cc. of serum was washed with a small volume of ammonium sulphate solution (20 g. salt; 100 cc. water) and the combined centrifugate and washings were dialysed. There were thus obtained 252 cc. of a yellow solution containing 3·6 g. solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·25 g. butyrylcholine chloride; 10 cc. of the enzyme solution were diluted to 15 cc., when 2 cc. of this solution required 7·85 cc. alkali in 20 mins. Volume-number, 742; weight-number, 164. Corresponding numbers for serum, 830, 99.

The above results are not considered to be sufficiently accurate to warrant applying corrections for the hydrolysis of the substrate by hydroxyl ions. It is nevertheless clear that the protein precipitated by 20 g. of ammonium sulphate per 100 cc. of serum contains little if any enzyme. By removing this protein a considerable purification is therefore effected. Calculated on the dry-weight, the activity is approximately doubled.

105 g. of ammonium sulphate were dissolved in 300 cc. of serum and, after standing for some hours, the solution was centrifuged. The liquid was rejected. The precipitate was dissolved in water and the solution dialysed. Some protein which separated during dialysis was removed in the centrifuge, when 168 cc. of solution were obtained. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 7·1 cc. alkali in 20 mins. Volume-number, 398 (serum, 465). A large proportion of the enzyme is thus precipitated under these conditions. In view of the fact that a slight loss of material occurred in this experiment owing to a leak in one of the collodion membranes, the following experiment was carried out.

The precipitate obtained by dissolving 40 g. of ammonium sulphate in 200 cc. of serum was removed and washed as described above. The ammonium sulphate content of the combined centrifugate and washings was increased to 35 g. per 100 cc., the precipitate removed in the centrifuge, dissolved in water and dialysed. 83 cc. of solution were obtained containing 11 g. of solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 9·5 cc. alkali in 20 mins. Volume-number, 369; weight-number, 67. Corresponding numbers for serum, 465, 55. It is clear that some enzyme escaped precipitation and that little purification is effected under these conditions.

A further 200 cc. of serum were treated with 40 g. of ammonium sulphate

and the precipitate was washed as described above. The ammonium sulphate content of the combined centrifugate and washings was increased to 38 g. per 100 cc. and sufficient acetic acid was added to render the solution slightly acid to litmus. The precipitate was removed, dissolved in water, and the solution dialysed. There were thus obtained 80 cc. of solution containing 8·4 g. of solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 9·7 cc. alkali in 20 mins. Volume-number, 388; weight-number, 116. Corresponding numbers for serum, 465, 55.

40 g. of ammonium sulphate were dissolved in 100 cc. of serum, the precipitate dissolved in water and the solution so obtained dialysed. The salt-free solution measured 65 cc. and contained 8 g. of solid. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 7·2 cc. alkali in 20 mins. Volume-number, 468; weight-number, 58. Corresponding numbers for serum, 465, 55.

The above experiments demonstrate that the choline-esterase is precipitated with the more soluble proteins. Beyond the purification effected by the removal of the virtually inactive precipitate obtained with 20 g. of ammonium sulphate per 100 cc. of serum, simple fractional precipitation with this salt is evidently useless as a method for increasing the purity of the enzyme.

Glycerol extraction. The 65 cc. of solution obtained in the last experiment of the preceding section were evaporated to dryness at a temperature not exceeding 30°. The solid residue (8 g.) obtained was ground to a fine powder and extracted for 1½ hours at 30° with 90 % glycerol. Undissolved solid was removed in the centrifuge and the solution poured into a collodion membrane and dialysed. The residue was dissolved in water and similarly dialysed. The solution obtained from the extract measured 27 cc. and contained 0·11 g. of solid per cc. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 6·55 cc. alkali in 20 mins. Weight-number, 60; serum weight-number, 55. The residue yielded 24 cc. of solution containing 0·086 g. solid per cc. Estimation: 1 cc. required 5·7 cc. alkali in 20 mins. Weight-number, 66.

Extraction with ammonium sulphate. A quantity of serum was treated with 20 g. of ammonium sulphate per 100 cc., and, after removal of the inactive precipitate, dialysed. The solution so obtained was evaporated to dryness at a low temperature and the residue ground to a fine powder. 2 g. of the latter were extracted with 10 cc. of ammonium sulphate solution (20 g. salt; 100 cc. water). After centrifuging, the solution and residue were dialysed separately. The former gave 20 cc. of solution containing 6·4 g. solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·25 g. butyrylcholine chloride; 1 cc. required 9·0 cc. alkali in 20 mins. Weight-number, 140; serum weight-number, 99. The solution obtained from the precipitate measured 9 cc. and had little activity: 1 cc. required 1·35 cc. alkali in 20 mins.

A further 2 g. of the solid were extracted for 1 hour with 10 cc. of ammonium sulphate solution (35 g. salt; 100 cc. water). The mixture was then centrifuged and the solution and residue were dialysed separately. The solution yielded

17·5 cc. of liquid containing 1·9 g. solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·25 g. butyrylcholine chloride; 1 cc. required 5·35 cc. alkali in 20 mins. Weight-number, 282; serum weight-number, 99. The solution from the precipitate measured 19 cc. and contained 7·4 g. solid per 100 cc. Estimation: 1 cc. required 8·0 cc. alkali in 20 mins. Weight-number, 108.

Final method of purification.

The best preparation of choline-esterase which we have hitherto obtained has been prepared by a method based upon the preceding experiments. In this method we have utilised the observations that the protein precipitated with 20 g. of ammonium sulphate per 100 cc. contains little or no enzyme, and that extraction with a solution of 35 g. of ammonium sulphate in 100 cc. of water of the residue obtained by evaporation of the dialysed centrifugate gives a solution in which the ratio of enzyme to protein, as reflected by the weight-number of the preparation, is much increased. Unfortunately, however, the process of evaporation, even when carried out under extremely mild conditions, is accompanied by some destruction of the enzyme. We have therefore attempted to avoid evaporation by the following procedure, which also possesses the advantage that dialysis of large volumes of solution is avoided. Since this procedure has provided us with our best preparation of choline-esterase and has given consistent results with different specimens of serum, a typical experiment is described in detail.

Ammonium sulphate (20 g.) was dissolved in 100 cc. of serum. The precipitate was removed in the centrifuge, washed with a small volume of a solution of ammonium sulphate (20 g. to 100 cc. water) and the mixture again centrifuged. The concentration of ammonium sulphate in the combined centrifugates, which measured 115 cc., was increased to 40 g. per 100 cc. by the addition of 23 g. of the solid salt, and the solution rendered faintly acid to litmus by the addition of 8 cc. of $N/2$ acetic acid. After about an hour, the precipitate which formed was centrifuged off, the liquid being rejected. The precipitate was then stirred in the centrifuge-glasses with about an equal volume of a solution of 35 g. of ammonium sulphate in 100 cc. of water. After standing overnight, the suspension was again centrifuged and the clear liquid poured into a flask. In the course of a few hours a crystalline precipitate, probably of albumin, separated. This was removed, and the concentration of ammonium sulphate in the liquid, which measured 34 cc., was increased to 40 g. per 100 cc. by the addition of 1·7 g. of the solid salt; 0·5 cc. of $N/2$ acetic acid was also added to render the solution faintly acid. A precipitate separated; after a few hours this was removed, dissolved in a small volume of water, and the solution so obtained was dialysed. A small quantity of protein separated during dialysis. This was centrifuged off and rejected, leaving a clear solution with a faint brown colour. Estimation: p_H, 8·8; substrate, butyrylcholine chloride (0·25 g.); 1 cc. of solution, which contained 1·996 % of solid, required 12·85 cc. of alkali in 20 mins. Weight-number, 644; serum weight-number, 99.

When a correction is applied for the hydrolysis of the substrate by hydroxyl ions, these figures become 581 and 82 respectively. This indicates that, based on the dry-weight, the purified preparation was seven times as active as the original serum. The purification actually effected was, however, probably greater than this, for, as previously explained, some uncertainty exists regarding the accuracy of the estimations made with unpurified serum. The exact degree of purification is, however, unimportant. The advantage of the process lies in the fact that the contents of protein and of yellow pigment in the preparation have been reduced to such an extent that the activity of the enzyme can be followed with great accuracy.

Action of choline-esterase on various substrates.

The activity towards various substrates of the preparation of choline-esterase described above was examined at two different acidities, with the results shown in Table I. 1 cc. of the enzyme solution was used in each case.

Table I.

Substrate	p_H	cc. 0·0225 *N* NaOH used in 5 min. periods	Total	Non-enzymic hydrolysis	Total (corr.)
Butyrylcholine	8·8	3·25, 3·2, 3·2, 3·2	12·85	1·35	11·5
,,	7·3	3·25, 3·3, 3·0, 3·2	12·75	0·1	12·65
Acetylcholine	8·8	2·2, 2·05, 2·1, 2·05	8·4	2·4	6·0
,,	7·3	1·4, 1·5, 1·35, 1·3	5·55	0·2	5·35
Methyl butyrate	7·3	0·15, 0·2, 0·2, 0·15	0·7	0·1	0·6

As substrates, 0·25 g. of the chlorides of the choline esters and 0·25 cc. of methyl butyrate were employed. The indicators used were *o*-cresolphthalein and bromothymol blue. Corrections for the non-enzymic hydrolysis of the substrates were determined under conditions identical with those used in the enzyme experiments. In addition to these experiments, the action of 1 cc. of the enzyme preparation on tributyrin was examined stalagmometrically under the conditions (p_H, 7·9; T., 20°) described by Stedman and Stedman [1931]. The diminution in the drop number in 40 mins. was 22.

The results with another preparation are given in Table II.

Table II.

Substrate	p_H	cc. 0·0225 *N* NaOH used in 5 min. periods	Total	Non-enzymic hydrolysis	Total (corr.)
Butyrylcholine	8·6	1·0, 1·05, 1·1, 1·1	4·35	0·65	3·7
,,	7·4	1·15, 1·15, 1·15, 1·15	4·6	0·1	4·5
Acetylcholine	8·6	1·25, 1·25, 1·1, 1·2	4·8	1·2	3·6
,,	7·4	0·8, 0·85, 0·8, 0·8	3·25	0·15	3·1

In these experiments the enzyme preparation was diluted in order to reduce the rate of hydrolysis of the substrates, which was somewhat too great in the experiments of Table I. Owing to the slower rate at which it was necessary to add the alkali, this permitted a much more accurate control of p_H.

For comparison with the results of Tables I and II, the experiments carried out with 1 cc. of the serum from which the preparations used above were made are recorded in Table III.

Table III.

Substrate	p_H	cc. 0·0225 *N* NaOH used in 5 min. periods	Total	Non-enzymic hydrolysis	Total (corr.)
Butyrylcholine	8·8	2·3, 1·9, 2·1, 2·0	8·3	1·35	6·95
Acetylcholine	8·8	1·7, 1·7, 1·6, 1·45	6·4	2·4	4·0
Methyl butyrate	8·8	0·6, 0·55, 0·45, 0·6	2·2	0·2	2·0

Using tributyrin as substrate with 1 cc. of the same specimen of serum, the diminution in the drop number (p_H, 7·9; T., 20°) in 40 mins. was 23. While great accuracy cannot be claimed for the results in Table III, they are reproduced because they show that the relative activities of the enzyme preparation towards the three types of substrate employed, namely, esters of choline, methyl butyrate and tributyrin, have changed considerably during the process of purification. In particular, the activities towards methyl butyrate and tributyrin are much depressed in the purified preparation. This may indicate that different enzymes are involved, but the data at present available are insufficient to permit of a definite decision on this point.

Summary and discussion.

The preceding experiments have demonstrated that there is present in the blood-serum from the horse an enzyme, for which the name choline-esterase is suggested, which hydrolyses both acetyl- and butyryl-choline, the latter substrate being attacked more rapidly than the former. This enzyme is clearly different from the liver-esterases from the pig and the cat, since the latter are without action on esters of choline. Methods for the purification of choline-esterase are described.

The question of the distribution of the new enzyme has not yet been examined. It must not, however, be concluded from the above results that it is necessarily absent from the liver. The liver-esterase which we have employed has been obtained by extracting desiccated liver powder with dilute ammonia, desiccation of the liver having been effected by the use of acetone and ether. It is probable that such treatment would destroy choline-esterase. Thus, in some preliminary experiments not recorded above an attempt was made to purify the enzyme in horse-serum by precipitating it together with the proteins with acetone and then drying the precipitate by successive treatment with this solvent and ether. The dry preparation so obtained dissolved completely in water, but the resulting solution was without action on acetylcholine.

The presence in horse-serum of an enzyme which hydrolyses tributyrin has long been known. As far as we are aware, however, its action towards simple esters such as methyl butyrate has not hitherto been investigated. The above results now show that this ester, in addition to tributyrin and esters of

choline, is hydrolysed by an enzyme present in the serum. Whether one enzyme is responsible for the hydrolysis of all three types of substrate, or whether different enzymes are involved, cannot yet be decided. That the relative activities of purified preparations of the enzyme towards the three substrates should prove to be different from that of serum must, however, be regarded as evidence in favour of the latter view.

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CCXLVI. VITAMIN C AND THE SUPRARENAL CORTEX.

I. ANTISCORBUTIC ACTIVITY OF OX SUPRARENAL.

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The chemical study of vitamin C has been notably advanced by the discovery that hexuronic acid possesses antiscorbutic properties. It will be recalled that when he first isolated hexuronic acid, Szent-Györgyi [1928] concluded that it was identical with the reducing substance which is found in active vitamin C concentrates. However, observations by Zilva seemed to show that the reducing substance in question bore no relationship to vitamin C, and hence the matter was dropped for some years. In the meantime a series of detailed investigations by Tillmans and his collaborators [1930; 1932] led to the conclusion that antiscorbutic activity went strictly parallel with reducing capacity and it was argued that the reducing substance was "the carrier of vitamin C activity." Recently the whole question has been re-opened by Svirbely and Szent-Györgyi [1932, 1, 2], who reported that a specimen of hexuronic acid (derived from ox suprarenals) in doses of 1 mg. per day would protect guinea-pigs from scurvy over a 90-day test period. Independently King and Waugh [1932; see also Waugh and King, 1932] have found that a crystalline preparation of vitamin C which they isolated from orange juice possesses the chemical and physical properties of hexuronic acid. The conclusion which Szent-Györgyi draws from his test that "vitamin C is a single substance identical with hexuronic acid" is disputed by Zilva [1932, 1], although he admits [1932, 2] that specimens of hexuronic acid may contain some vitamin C. Zilva [1932, 3] opposes also the conclusions of Tillmans.

In a previous communication from this laboratory [Harris, Mills and Innes, 1932] confirmation was obtained, by means of striking curative tests and also by the microscopic tooth-structure method, of the conclusion which Svirbely and Szent-Györgyi had reached from a preventive test, that hexuronic acid possesses antiscorbutic potency; and its degree of activity was also determined quantitatively. The question was discussed whether the antiscorbutic action of hexuronic acid was an inherent property of the pure substance itself

or whether possibly it was due to traces of some associated impurity. It was pointed out that the theory that the vitamin and hexuronic acid are one and the same substance demands that the raw suprarenal cortex should possess an exceptionally high antiscorbutic activity, since this organ is known to be an even richer source of hexuronic acid than is orange juice itself. This we have found to be the case, the degree of activity of the cortex being in fact proportional to its high content of hexuronic acid[1]. A description of these experiments forms the subject of the present paper[2].

Experimental.

Supplies of fresh ox suprarenal were obtained daily or every second day from local slaughter-houses and used with as little delay as possible, being kept in the mean time in cold store in an atmosphere of CO_2. On removal from cold store, the medulla was dissected away from the cortex, and the latter, after being passed through a fine wire sieve, was suspended in water and immediately administered by means of a dropper to the guinea-pigs used as experimental animals. One object of feeding the cortex only and removing the medulla was to avoid any possibility of adrenaline poisoning. It was also desirable to administer the vitamin in as concentrated a form as possible (there is no evidence of any being present in the medulla) since earlier experience in this laboratory has shown that guinea-pigs fail to thrive if given too large amounts of animal tissue.

The antiscorbutic activity of the suprarenal cortex was determined (1) by curative tests, and (2) by the microscopic tooth structure method.

1. *Curative tests.*

This method has been used successfully in previous work published from this laboratory [*e.g.* Harris, Mills and Innes, 1932; Mills, 1932]. Matched guinea-pigs, males only, weighing about 250 g. are first placed for a preliminary period of about 10 days on the scorbutic basal diet:

Bran	80	parts by weight
Oats	720	„
Egg-yolk	40	„
Salts	8·4	„
Cod-liver oil	1	%

supplemented by 15 g. per day of cabbage, and next on the basal diet alone. Within a further period of 2 to 3 weeks animals are chosen for test which are showing early symptoms of scurvy, have begun to lose weight evenly and have dropped sharply 10 to 20 g. from their maximum weights (in the course of about 3 days) (see figure). Any animals whose weight curves have been lacking in smoothness or who have shown a departure from the average normal response are discarded, as also are those giving indications of scurvy complicated by infection (a not infrequent occurrence).

[1] Preliminary report [Harris, Mills and Innes, 1932].

[2] As reported in an earlier note [Dann *et al.* 1932] no confirmation could be found for the claim of Rygh and co-workers [Rygh and Rygh, 1932, 1; Rygh, Rygh and Laland, 1932] to have identified vitamin C with methyl*nor*narcotine or irradiated narcotine, and the criticisms there made of their technique and their deductions do not seem to be met by their later communication [Rygh and Rygh, 1932, 2].

Graded daily doses of the antiscorbutic supplement are then fed, and the recovery rates noted. A series of graded doses of orange juice (standard) is fed at the same time to another set of animals[1]. Negative controls are also taken and permitted to stay on the scorbutic diet, to

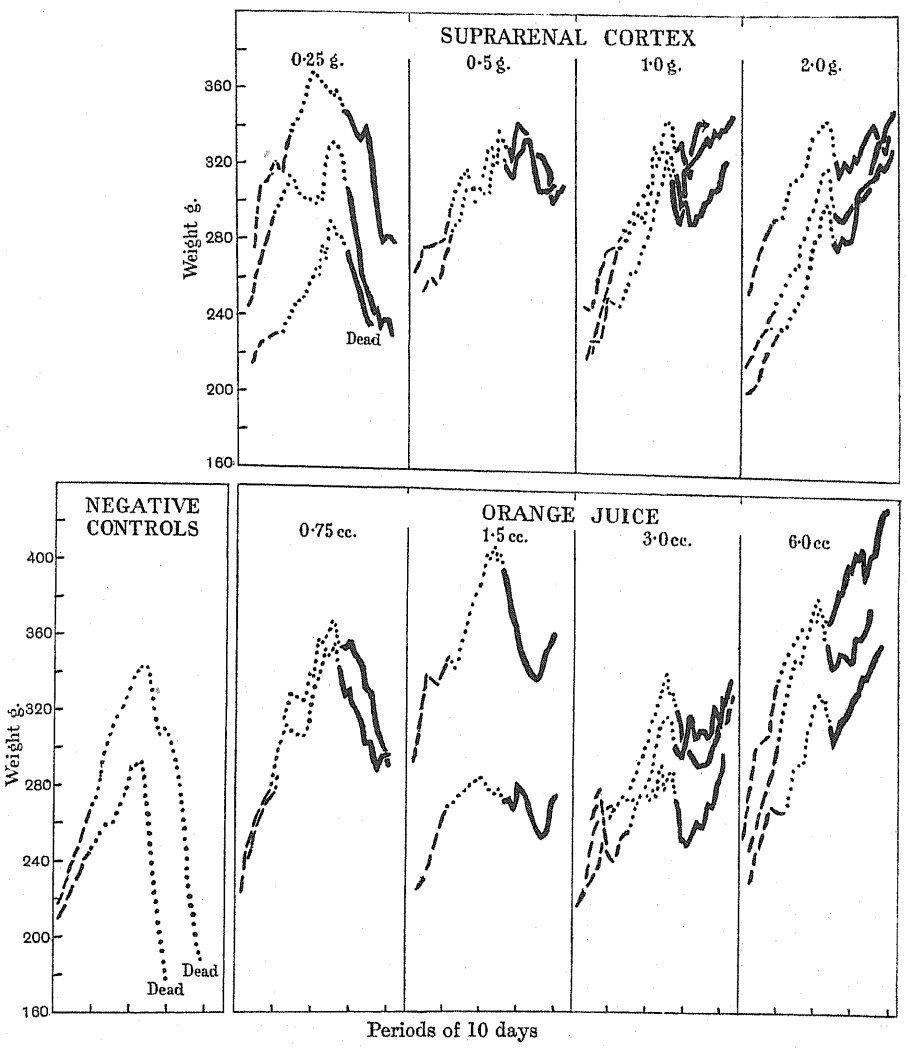


Fig. 1. Weight curves of guinea-pigs, comparing degrees of cure of scurvy with graded doses of suprarenal cortex (above) and orange juice (below).

— — — Preliminary complete synthetic diet.
..... Vitamin C-free diet. ═══ Experimental period.

demonstrate that in a given batch or litter the animals are behaving regularly, and would, in fact, continue to lose weight rapidly and die from scurvy but for the addition of the antiscorbutic supplement. After a day or two, growth is resumed at full rate, provided adequacy of vitamin C has been allowed. With partial adequacy subnormal growth results (see Fig. 1), or the decline in

[1] Like Key and Elphick [1931], we have found orange juice preferable as standard of reference to lemon juice (the International Standard).

weight is merely stayed. Thus one can compare the doses of unknown and of standard needed to cause equal resumption in growth rate.

This method possesses a number of advantages. The demonstration of antiscorbutic activity is dramatic and unequivocal. The method is also sparing of material and is of course more rapid than the usual preventive method, since, assuming a continual supply of guinea-pigs developing scurvy, a test can safely be concluded within as short a period as 10 days. Practically the only precaution is to make no use of animals which have declined too far, and would therefore fail to respond. Animals which develop intercurrent infection or other complication to the scurvy, which would likewise prevent a normal response, should be omitted from consideration.

In the present determinations, the suprarenal cortex was tested at four levels, 0·25, 0·5, 1·0 and 2 g., and the results compared with those given by orange juice fed at the four levels of 0·75, 1·5, 3 and 6 cc. Three animals were included in each of these eight groups except that at the two lower levels of orange juice and one on suprarenal cortex only two could be spared. The individual growth curves are shown in Fig. 1. Fig. 2 gives the average gains in weight plotted against the amounts of antiscorbutic material fed.

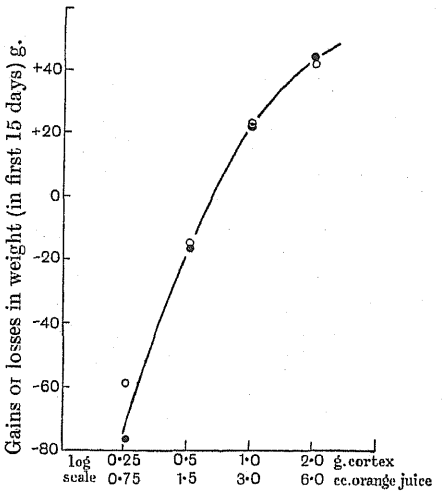


Fig. 2. Vitamin C dosage-response curve, showing average weight gain or loss with given doses of supplements.

○ Orange juice. ● Suprarenal cortex.

Result. It will be seen that 2·0 g. of raw suprarenal cortex corresponded very accurately with 6 cc. of orange juice, 1 g. with 3 cc., and 0·5 g. with 1·5 cc.

2. *Tooth-structure method.*

This method, based on Höjer's work, and depending on preventive action on microscopic tooth structure, has been used with satisfaction in this laboratory for the past 4 years. Normal young guinea-pigs weighing about 300 g. are given the scorbutic basal diet cited above supplemented with suitable graded daily doses of the substance to be tested. Reference animals receive graded doses of orange juice. After 14 days all the animals are killed, and cross sections of the roots of the incisors are prepared by the usual method. Comparison is made between the appearance of the teeth in the groups receiving unknown and standard (orange juice); preferably the amounts may be compared which just suffice to permit the development of normal tooth structure.

In a preliminary experiment raw suprarenal cortex was tested at levels of 0·5, 1·0 and 2·0 g., using three animals at each level. For reference orange juice was fed at levels of 1·5, 3 and 6 cc. Negative controls were given the basal diet alone.

The result of this test (Table I) showed that the minimum dose of suprarenal cortex needed for complete dental protection was approximately 1·0 g., compared with 3 cc. for orange juice.

Table I.

Material tested	Daily dose	Degree of protection on Key and Elphick's [1931] scale ("4" represents full protection)
Suprarenal cortex	0·5 g.	2, 2, 1 (av. 2)
,,	1·0 g.	4, 4, 4 (av. 4)
,,	2·0 g.	4, 4, 4 (av. 4)
Orange juice	1·5 cc.	3, 2 (av. 2–3)
,,	3·0 cc.	4, 4 (av. 4)
,,	5·0 cc.	4, 4 (av. 4)
Negative control	(No supplement)	0, 0 (av. 0)

In a confirmatory test 5 guinea-pigs were each given 0·75 g. of suprarenal cortex daily, an amount judged to be sufficient to produce a moderate degree of protection. Reference animals had 2 cc. of orange juice. The results (Table II) are in keeping with the two earlier assays.

Table II.

Material tested	Tooth sections, degree of protection in individual animals
Suprarenal cortex, 0·75 g. daily	3, 2, 2–3, 1, 2 (av. 2–3)
Orange juice, 2·0 cc. daily	2–3, 2 (av. 2–3)
Negative control (basal diet only)	0–1, 0–1 (av. 0–1)

Experiments with guinea-pig suprarenals.

Preliminary experiments on normal guinea-pig suprarenals show them to be comparable with ox suprarenals in vitamin C activity. Suprarenals from scorbutic guinea-pigs on the other hand were devoid of vitamin C (see Table III). Fuller details will be published in a later paper.

Table III.

Diet	Weight gains or losses during first week of curative test (g.)
Basal diet alone (negative control)	−72, −39
Suprarenal glands (whole) from scorbutic guinea-pigs, 0·5 g. per day	−81, −82
,, ,, normal ,, ,,	+15, −39

Discussion.

1. *Vitamin C and hexuronic acid.*

The first conclusion emerging from our results is that the relative antiscorbutic activities of suprarenal cortex and orange juice are exactly proportional to the amounts of hexuronic acid which can be isolated from these

two sources. According to Szent-Györgyi [1928], three times as much hexuronic acid can be recovered from a given weight of suprarenal cortex as from orange juice (300 mg. and 100 mg. per kg., respectively), and our experiments show that raw suprarenal cortex possesses also precisely three times the antiscorbutic activity of orange juice. This result is therefore in excellent conformity with the theory (although of course not conclusive evidence for it) that vitamin C is identical with hexuronic acid, assuming the same degree of recovery of hexuronic acid from the two sources.

Actually it is easy to show that a considerable loss of antiscorbutic material occurs during the course of isolation of the hexuronic acid in both cases. Thus, from 1 kg. of suprarenal cortex 300 mg. of hexuronic acid were isolated [Szent-Györgyi, 1928], representing according to the results of Harris, Mills and Innes not more than 300 minimum protective doses (Höjer), as compared with about 1000 doses in the original kg. of cortex itself. The loss is therefore fully two-thirds. The percentage loss is presumably identical with that from orange juice, the hexuronic acid recovered from the two sources possessing the same antiscorbutic activity (see below). While admittedly, the evidence for identifying vitamin C with hexuronic acid would have been more conclusive had the whole of the antiscorbutic activity of the original raw material been successfully concentrated in the form of the hexuronic acid separated from it, it is nevertheless too much to expect that no loss should occur when working with so unstable a material as vitamin C; and the fact that the percentage loss is the same when the same process is applied to widely different raw materials might be regarded as evidence for rather than against the theory. Furthermore the loss of vitamin seems to run parallel with that of hexuronic acid, since Szent-Györgyi [1927; 1928] estimates the amount of the latter actually present in the cortex to be two or three times that which can be recovered from it.

It is also possible to account approximately for the antiscorbutic activity of the cortex, or of orange juice, on the basis of its total hexuronic acid content, estimated chemically. For cortex, this is from 600 to 1000 mg. per kg. according to Szent-Györgyi's [1927; 1928] data; say 1000 mg. Now the minimum (Höjer) dose of hexuronic acid is from 1 to 2 mg. according to the findings of Harris, Mills and Innes; say 1 mg. Hence there are 1000 doses of 1 mg. each in the 1000 mg. of hexuronic acid in the 1 kg. of cortex. That is, the minimum dose of cortex is 1 g. This agrees with our experimental finding. But it is only by thus choosing maximum values both for the hexuronic acid content and for the activity of the hexuronic acid, that values in such good accordance with observation are reached. (This applies also in calculating the activity of other sources from their hexuronic acid contents.) The choice of the maximum value for the activity of hexuronic acid may be justifiable since it is possible that the observed value is low on account of the difficulty of preventing some inactivation. Again it seems probable that the data for hexuronic acid contents as originally given by Szent-Györgyi were under-

estimated. Thus, in good conformity with the hexuronic acid theory, we have found that the titration values of hexuronic acid and of orange juice as determined under suitable conditions by means of the reduction indicator, 2:6-dichlorophenolindophenol [Tillmans, 1930], are proportional to their relative antiscorbutic activities.

Vitamin history, however, shows the danger of assuming without the most compelling evidence that the activity of an apparently pure material is necessarily due to the main constituent and not to some unsuspected impurity. Now if one supposes the pure vitamin C to have a minimum dosage at all comparable with that of the other vitamins (D, A or B_1), it follows that the activity of hexuronic acid must be due to traces of some associated impurity, amounting to no more than a small fraction of 1 %, the presence of which could easily be overlooked. It will be admitted however that there is no *a priori* justification for arguing the dosage from analogy with the other vitamins. And, while giving full weight to the above considerations, the existing evidence seems to contain nothing definitely discordant with the theory that vitamin C is identical with hexuronic acid; while, speaking more positively, it seems hard to escape the conclusion that the vitamin if not actually hexuronic acid itself is at any rate a substance possessing a close similarity to it both in its distribution and in its chemical behaviour. Whatever the final solution to the problem, this undoubtedly represents an important advance.

The evidence for the above conclusion may be summarised briefly as follows. (1) Vitamin C so far as is known has a similar distribution in nature, to hexuronic acid (*viz.* in suprarenal cortex, orange juice, cabbage[1], and (?) lemon juice). (2) The specimen of hexuronic acid from suprarenals examined by us had the same degree of antiscorbutic activity as those derived from vegetable sources, tested elsewhere. (3) The widely different processes used for the isolation of (*a*) hexuronic acid by Szent-Györgyi and (*b*) vitamin C by King gave a final material possessing identical antiscorbutic activities and, apparently, chemical and physical properties. (4) Vitamin C closely follows hexuronic acid in the various precipitation and solubility reactions used in the isolation processes. (5) None of the known properties of the antiscorbutic factor is inconsistent with it being hexuronic acid. (6) As pointed out by Szent-Györgyi, Tillmans' observations on the association of antiscorbutic activity with reducing capacity are explicable on the hexuronic acid theory. Svirbely and Szent-Györgyi [1932, 2] have offered an explanation of Zilva's apparently contradictory findings in this direction. Zilva's objection [1932, 1], that he has obtained concentrates with greater activity than hexuronic acid, has been replied to by Svirbely and Szent-Györgyi [1932, 2].

[1] Szent-Györgyi [1928] isolated 250 mg. of hexuronic acid from 5 kg. of cabbage. Making an allowance for his acknowledged greater loss with this material the yield is in good agreement with that calculated from the antiscorbutic potency in comparison with orange juice and suprarenal. Lemon juice has the same antiscorbutic activity as orange juice and apparently a similar content of hexuronic acid although it would seem that the precise figure for the latter has not previously been determined.

The evidence of the present paper is of a remarkable degree of parallelism between hexuronic acid and antiscorbutic activity. The question can only be finally settled by further work showing whether this parallelism is invariable and complete.

2. Vitamin C and the physiology of the suprarenals.

Our results show that the suprarenal cortex possesses an astonishingly high antiscorbutic activity, hitherto quite unsuspected. The organ is considerably more potent in vitamin C than any natural source hitherto known, and certainly contains the vitamin in far greater concentration than any other animal tissue so far investigated. The liver only [Mills, 1932] may contain a greater total quantity of vitamin C, by virtue of its large bulk, but it does not compare with the suprarenal so far as localised concentration is concerned.

What is the significance of the unique collection of vitamin C in this site? The possibility suggests itself that as the liver may serve as a storehouse for vitamin C, so the suprarenal may perhaps be concerned in its elaboration or utilisation. On the other hand, it seems likely that the vitamin may be needed by the suprarenal for its functional activities—as in maintaining adrenaline-like substances in a reduced condition. One of us [Harris, 1931] has shown that the dog, unlike the guinea-pig, monkey or man, is able to synthesise its own vitamin C when none is present in the diet, and the same appears to apply to many other species, including the rat [Parsons, 1920]. Tests are in progress to determine the behaviour of the suprarenal and whether it continues to act as a source of vitamin C in such species under these conditions. We have shown that in the guinea-pig, which by contrast is unable to synthesise its own vitamin C, the suprarenal loses its antiscorbutic activity coincidently with the onset of scurvy. The idea that the suprarenal has a specially intimate physiological connection with vitamin C gains support from the observation [McCarrison, 1920] that it undergoes highly characteristic hypertrophic changes in scurvy.

SUMMARY.

Raw suprarenal cortex (ox) is shown to be a more powerful antiscorbutic than any natural source hitherto known, possessing three times the activity of fresh orange juice (or 30 International Units per g.).

The degree of activity corresponds precisely with the value predicted on the basis of the relative yields of hexuronic acid obtainable from the two sources. (There is however a considerable loss of antiscorbutic material (the same in the two cases, *viz.* over 50 %) and of hexuronic acid during the course of the isolation.) The antiscorbutic potency of the cortex can also be accounted for on the basis of its total hexuronic acid content, as estimated chemically, provided certain assumptions are made in the calculation.

From the foregoing and other evidence it is concluded that vitamin C is either identical with hexuronic acid or is a substance possessing a close

similarity to it in its distribution and in its chemical nature, judging by its behaviour towards various solvents, precipitating agents, *etc.*

Preliminary results show that in guinea-pigs—a species which resembles man and monkeys but differs from many others, including dogs and rats, in being unable to synthesise vitamin C *in vivo* when none is provided in the food—the vitamin C activity of the suprarenal is lost with the onset of scurvy. It is supposed that vitamin C plays a special rôle in the physiology of the suprarenal. The occurrence of distinctive hypertrophic changes in the suprarenals in scurvy is in keeping with this.

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CCXLVII. THE IODIMETRIC TITRATION OF CYSTEINE AND ALLIED SUBSTANCES.

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The increasing interest of biochemists in sulphydryl compounds has led to the necessity of finding some method for the accurate determination of these substances. That the problem is complex may be judged from the number of papers proposing new methods or modifications of old ones. The original procedure of Rosenheim and Davidsohn [1904] for the volumetric estimation of thiol acids by the use of iodine in the presence of sodium bicarbonate was found by Klason and Carlson [1906] to give too high values. However, in neutral or acid solutions the reaction between sulphydryl compounds and iodine was shown to be quantitative. Nearly all subsequent iodimetric procedures are based on this observation.

The sulphydryl compounds of especial biochemical interest, cysteine, glutathione and ergothioneine, appear to be peculiarly sensitive to the acidity and temperature of the reaction mixture in which they are titrated. It has been known in a general way that cysteine is oxidised to cystine by iodine and to cysteic acid by bromine. The bromate and iodate methods for cysteine described by Okuda [1925] are based on these observations. Okuda showed that on adding standard bromate to an acid solution of cysteine containing excess of bromide, quantitative oxidation to the sulphonic acid occurred. The concentration of the bromide had no effect as long as it was in excess; the concentration of acid affected only the rate, but not the nature of the reaction, and temperature had almost no effect. However, in spite of the simple course of the reaction, the procedure is not suitable for use on protein hydrolysates, since tyrosine, histidine and tryptophan all react with bromine. Since cystine alone of the amino-acids reacts rapidly with iodine in acid solutions, Okuda studied a similar iodate method. The reaction is somewhat complex, a mixture of disulphide and sulphonic acid (and possibly other products) being formed. The temperature and concentration of acid in the mixture have a pronounced effect on the course of the reaction, which is influenced to a less degree by the concentration of iodide [Okuda, 1924] and other substances present. That this is also true of glutathione has been shown by King and Lucas [1931], who found the amount of iodine required by glutathione to be influenced by both temperature and acidity, and by Perlzweig and Delrue [1927], Gavrilescu [1931]

[1] Holder of the Dr James Page Rutherford Research Grant.

and others, who showed that the presence of added potassium iodide lowered the iodine consumption of glutathione solutions. In the hope of gaining some useful information concerning the conditions under which these compounds are most favourably titrated, a study has been made of the influence of acidity, temperature and concentration on the reaction between iodine and cysteine and several allied substances.

Experimental.

The sulphydryl compounds used in this investigation were dissolved in freshly boiled and cooled distilled water, and, unless otherwise stated, diluted to make approximately 0·04 *M* aqueous solutions. 1 cc. of solution was added to 11 cc. of buffer mixture made up with oxygen-free water. The buffers used were (1) Walpole's *M* sodium acetate + HCl, (2) the 0·1 *M* glycine-NaCl + HCl mixture of Sørensen, (3) Walpole's acetic acid + sodium acetate mixture, (4) McIlvaine's Na_2HPO_4 + citric acid, (5) Sørensen's NaH_2PO_4 + Na_2HPO_4 and (6) the sodium veronal buffer of Michaelis. In some of the work the Universal Buffer Mixture sold by British Drug Houses was used. After mixing the buffer and test solution the p_H was always checked by a capillator measurement before the titration was performed. From time to time the p_H was measured after the titration was completed, to make sure that the buffering action had been effective.

If no indicator is used in the iodimetric titration, a point is reached at which the iodine itself suddenly gives a yellow coloration to the solution. Okuda [1923; 1924; 1925] utilised this in his iodate method for cysteine. This end-point is sharp and readily reproducible, but the iodine used is always appreciably in excess of that required by theory if the reaction occurs according to the equation $2XSH + I_2 \rightarrow XSSX + 2HI$. The use of starch as indicator is not only much simpler, according to Uyei [1926], Blanchetière and Melon [1927] and Turner [1929], but appears to give more accurate results for glutathione. It has the disadvantage that in the case of cysteine and its derivatives, the end-point is not sharp—a pale purplish-blue, fading rapidly, precedes the intense blue characteristic of the starch-iodine complex, rendering doubtful the exact end-point. However, with a little experience, it was found possible to determine this point within 0·2 to 0·3 cc. in 10 cc., *i.e.* less than 3 % error. Tunnicliffe [1925] used nitroprusside as an external indicator in applying the iodine titration procedure of Klason and Carlson [1906] to the estimation of glutathione. Later, Kennaway and Hieger [1927] improved the nitroprusside indicator technique. To a series of small test-tubes they add 2 cc. saturated ammonium sulphate, 2 drops strong ammonia and 5 drops of fresh 1 % nitroprusside solution. 5 to 10 drops of the titration mixture are added to the indicator in the test-tubes. Procedures involving the use of outside indicators are not only troublesome but are scarcely suitable for a micro-method. Moreover as the quantity of thiol compound left in solution becomes small, the nitroprusside colour fails to develop proportionately. We

have found that in the case of glutathione, for example, when only 0·01 to 0·02 mg. per cc. is left in the reduced form, no colour at all appears. Hence a correction factor must be added when nitroprusside is used. The authors add to the observed quantity of glutathione 0·015 V mg., where V is the volume of solution at the apparent end-point. Such a correction factor has been noted by several workers, although many appear to have neglected to use it.

Cysteine.

The effects of temperature and p_H were first studied, since these factors exert the most marked influence on the iodine-combining power of cysteine. The potassium iodide factor was kept constant, either at the lowest possible level by using standard iodine instead of iodate, which requires the presence of an excess of iodide, or by adding a definite amount of iodide to the solution and titrating with iodine. Most of the figures were obtained by direct titration, but in some cases an excess of iodine was added to the cysteine solution and the determination completed by a back titration with 0·01 N thiosulphate. Both methods gave comparable results at 0°. The marked temperature effect when the titration was performed in N hydrochloric acid is shown in Fig. 1.

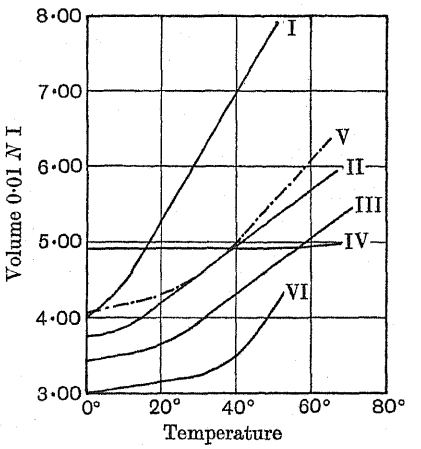


Fig. 1. Effect of temperature on the iodine consumption of sulphydryl compounds: cc. 0·01 N I combined by 1 cc. of (I) 0·04 M cysteine, (II) 0·037 M cysteine ethyl ester, (III) 0·034 M acetylcysteine ethyl ester, (IV) 0·049 M thiophenol, (V) 0·04 M thioglycollic acid, (VI) 0·03 M thiolactic acid.

Influence of acidity on iodimetric titration of cysteine. In order that the temperature of the test solution should not rise above 0°, the vessel was kept in a large dish of brine at about − 5° and the iodine added at a rate not exceeding 1 cc. per minute, the solution being stirred by means of a thermometer. The lowest iodine consumption, compatible with a reasonably fast rate of reaction, took place in solutions N with respect to strong mineral acid (hydrochloric). The reaction under these conditions, and at this dilution (1 cc. of 0·04 M cysteine in 12 cc.) conforms closely to the amount of iodine required

for oxidation to cystine, *i.e.* 4·00 cc.[1] The same quantity of cysteine in an equal volume of 0·1 *N* HCl required from 4·6 to 5·1 cc. of iodine. Although the end-point was sharper in the sense that a blue colour appeared more suddenly the values were quite variable and were not exactly reproducible. Decreasing the acidity by the use of various buffers led to surprising variations in the iodine-combining capacity of cysteine. In spite of careful temperature and p_H control, it was found impossible to get good checks, irregularities of from 0·1 to 0·6 cc. (depending upon the p_H) being apparently unavoidable. The results are best shown graphically (see Fig. 2).

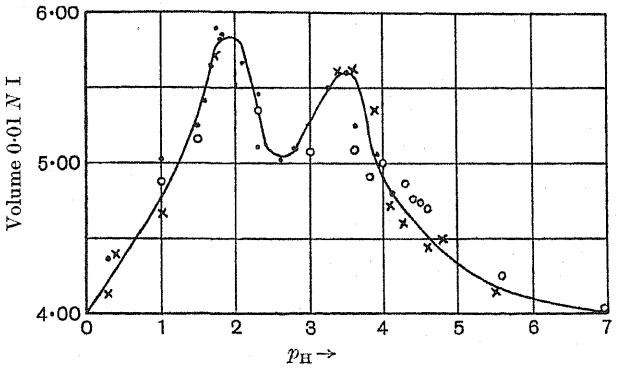


Fig. 2. Effect of p_H on the iodine consumption of cysteine: 1 cc. 0·04 *M* cysteine in a volume of 12 cc. titrated at 0°. (×) HCl and acetic acid-acetate buffers, (o) glycine, citrate and phosphate buffers, (•) B.D.H. Universal Buffer Mixture.

With decreasing acidity increasing amounts of iodine are required, until a maximum iodine consumption is reached at a p_H of about 1·8. The iodine requirement then falls to a minimum at p_H 2·7, rises to a second maximum at p_H 3·5, falls again to a minimum at p_H 7·0, and rises again on the alkaline side of neutrality. At first it was imagined that these peculiar titration values might be in some way related to the buffer solutions, but the same curve was obtained regardless of what buffers were utilised.

In solutions more acid than *N* the starch blue colour appears before the theoretical end-point, fades more or less rapidly, depending on the acidity of the medium, and is not readily determined by direct titration. Titration mixtures were set up as follows:

N: 1·0 cc. concentrated HCl + 1·00 cc. cysteine solution + 10·0 cc. water,
2*N*: 2·0 cc. concentrated HCl + 1·00 cc. cysteine solution + 9·0 cc. water,
3*N*: 3·0 cc. concentrated HCl + 1·00 cc. cysteine solution + 8·0 cc. water.

Because of the end-point difficulty the iodine was added dropwise to 4·0 cc. (the theoretical end-point), the apparent end-point being noted, and then run into 10·0 cc. After the acidity was adjusted (by adding the calculated extra

[1] This agreement may be fortuitous since, in the light of data presented in Table II, it would appear that at other dilutions different amounts of iodine are required.

amount of HCl) the mixtures were set aside at 0° in the dark for the times shown in Table I. From the amount of 0·01 *N* sodium thiosulphate required for back titration, the iodine consumption was calculated. The influence of increasing acidity is seen to be a slight slowing of the first reaction, namely, oxidation to disulphide and a marked inhibition of secondary reactions.

Table I. *Influence of high acidity on uptake of iodine by cysteine.*

HCl	I used at apparent end-point cc.	I used in 5 min. cc.	I used in 20 min. cc.	Increased I uptake in 15 min. cc.
N	4·05	4·20	4·75	0·55
2*N*	3·00	4·28	4·34	0·06
3*N*	2·2	3·91	3·91	0·00

Influence of cysteine concentration on iodimetric titration. The effect of dilution upon the iodine consumption of cysteine solutions has already been investigated by Bierich and Kalle [1928] in connection with their studies on the sulphydryl compounds in tissue extracts. By means of their so-called "dilution factor," which is very large for cysteine, they proposed to distinguish between cysteine and glutathione as iodine absorbers in such solutions. The object in the present investigation was not to devise a method of distinguishing sulphydryl compounds by this means, but to record the magnitude of the errors involved by dilution.

All titrations were performed at 0°, 0·01 *N* iodine being added dropwise to the well-cooled mixture containing 1 cc. of 0·04 *M* cysteine solution in a volume *V* (which initially was *N* with respect to HCl). The theoretical titration was 4·00 cc. The values obtained are contained in Table II.

Table II. *Influence of cysteine concentration on uptake of iodine.*

Constant quantity of cysteine in varying volumes.

Volume cc.	0·01 *N* I used cc.
8	3·9
12	4·0
16	4·4
24	4·8
32	5·0

Table III. *Influence of cysteine concentration on iodine consumption.*

Constant volume, varying amounts of cysteine.

Vol. of cysteine solution in total vol. of 12 cc., cc.	Volume 0·01 *N* I Required cc.	Theory cc.	Difference cc.
1·00	2·7	2·3	0·4
2·00	4·9	4·6	0·3
3·00	7·2	6·9	0·3
4·00	9·3	9·2	0·1

A somewhat similar series with the volume constant and varying cysteine concentrations gave similar results as shown in Table III. Varying volumes of

a cysteine solution containing 0·023 milli-equivalents per cc. were made up to a final volume of 12·0 cc., so that the acidity with respect to HCl was *N*. It is seen that the stronger the cysteine solution, the less is the extent to which iodine in excess of the theoretical is consumed.

The influence of iodine concentration. All titrations were performed at 0° on solutions *N* with respect to HCl, and containing 1 cc. 0·04*M* cysteine solution in an initial volume of 12 cc. Iodine of different strengths was added dropwise to the well-cooled solutions. The theoretical titration was 4·00 cc. 0·01*N* I. The values determined are contained in Table IV, where it is seen that the departure from the theoretical consumption is greater when standard iodine solutions of low normality are used.

Table IV. *Influence of iodine concentration on titration of cysteine.*

Normality of I used	Volume I: Required cc.	Volume I: Theory cc.	Difference: cc.	Difference: %	Difference: As 0·01 *N* I cc.
0·010	4·03	4·00	—	—	—
0·008	5·50	5·00	0·50	10	0·4
0·006	7·80	6·66	1·14	17	0·6
0·004	11·90	10·00	1·90	19	0·8

From a consideration of Tables II, III and IV, it is evident that dilution of either the cysteine solution or the iodine introduces errors which increase with the dilution, but not in any exact ratio to it. In stronger solutions oxidation to the disulphide only occurs, but at the dilutions at which protein hydrolysates and tissue extracts are titrated, the side-reactions introduce a considerable error.

Order of addition of reagents. The influence of the order of addition of reagents was studied briefly. Iodine was added in excess to cysteine solutions and in other cases cysteine was added to excess iodine solution, but although the amounts of iodine combined at different hydrogen ion concentrations were altered, in neither case were the results much closer to the stoichiometric relationship than by direct titration. The result of adding a considerable excess of iodine to the cysteine solution at 0° and various acidities followed by back titration with thiosulphate is shown in Fig. 3. The results in the acid range are uniformly lower than those obtained by direct titration. The first maximum of iodine consumption (at p_H 1·8) is completely obliterated, whereas the second maximum (at p_H 3·5) remains. Iodine consumption falls to a minimum at p_H 6 and rises fairly sharply with increasing p_H, much more iodine being combined at p_H 7 than is the case with direct titration.

Influence of potassium iodide on iodimetric titration of cysteine. Okuda's [1925] observation, that in his iodate method the presence of an excess of potassium iodide lowered the iodine consumption of cysteine solutions, led to an investigation of its influence in the hope that side-reactions might be entirely inhibited. Because of the premature development of the blue colour

in the presence of added iodide, it was found expedient to add an excess of iodine to the cysteine solution and to titrate the excess with thiosulphate. The marked effect of added iodide in lowering the iodine consumption, particularly at acidities less than N, is shown in Fig. 3: 0·5 % potassium iodide partially eliminates the second maximum of iodine consumption at p_H 3·7, and 2 % iodide almost completely.

It is perhaps worthy of note that the shape of the curve relating iodine consumption to p_H for cysteine differs markedly from that for a trichloroacetic acid extract of liver, the iodine consumption of which is constant between p_H 0 and 5, as was shown by King, Baumgartner and Page [1930]. The curve for liver extracts is similar in shape to that for glutathione, and the absence of any

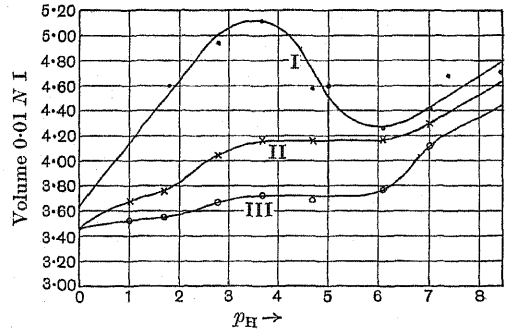


Fig. 3. Influence of p_H on the indirect titration of cysteine: excess iodine added to 12 cc. buffered cysteine hydrochloride solution at 0°, containing (I) no added KI, (II) 0·5 % added KI, and (III) 2 % KI. Excess iodine titrated with 0·01 N sodium thiosulphate.

peaks from the curve, similar to those which are so characteristic of cysteine, would appear to indicate the absence of appreciable amounts of free cysteine from liver.

Secondary oxidation products. The nature of the products formed by the iodine used in excess over that required for oxidation to disulphide, has not been investigated fully, but by allowing a series of tubes containing cysteine to stand for some days with twelve times the iodine necessary for oxidation to disulphide (or twice that necessary for oxidation to cysteic acid) and titrating with thiosulphate at intervals, evidence of eventual oxidation to cysteic acid was obtained. It was found that after 50 hours at room temperature the iodine consumed by cysteine was 92 % of that required by theory for oxidation to cysteic acid. Data from a similar experiment with cystine, as shown in Table V, demonstrated that the oxidation to cysteic acid proceeded to the same point in 50 hours and to 94 % of completion in 100 hours. Since this work has been completed, a paper has appeared by Shinohara [1932] in which the isolation of cysteic acid from the oxidation products of cystine by iodine has been described.

The data set forth in the preceding sections make it plain that no simple rules can be laid down which will ensure quantitative results for the estimation

Table V. *Rate of oxidation of cystine by excess of iodine.*

4·8 mg. cystine in 5 cc. water + 5·0 cc. 0·10 *N* I and 1 cc. conc. HCl, temperature 20°.

Time hours	0·01 *N* $Na_2S_2O_3$ cc.	0·01 *N* I used cc.
0	51·2	0·00
1	47·0	4·2
2	45·7	5·5
4	43·2	8·0
8	39·0	12·2
24	33·2	18·0
50	32·9	18·3
101	32·4	18·8
		Theory 20·0

of cysteine in solutions of unknown strength. Temperature, acidity, concentration (both of the cysteine and of the iodine), the rate and order of addition of reagents and the presence of potassium iodide in varying amounts all affect the iodine consumption in variable ways and to different degrees. Probably the conditions which give results most uniformly according with theory over a wide range of concentration, are indirect titration at 0°, *N* acidity with respect to strong acid and the presence of 0·5 % potassium iodide. Table VI contains the results of an experiment in which cysteine at different dilutions was titrated under these conditions. The results are probably as good as can be obtained under any single set of conditions for the iodimetric titration of cysteine.

Table VI. *Titration of cysteine in different dilutions at 0°, N acidity and 0·5 % KI concentration.*

1 cc. *M*/12 cysteine hydrochloride in different volumes.

Volume cc.	0·1 *N* I cc.	0·1 *N* $Na_2S_2O_3$ cc.	0·1 *N* I combined cc.
1	2·00	1·200	0·800
2	2·00	1·200	0·800
5	2·00	1·200	0·800
10	2·00	1·210	0·790
20	2·00	1·200	0·800
50	2·00	1·205	0·795
100	2·00	1·205	0·795

X cc. *M*/12 cysteine hydrochloride in a volume of 50 cc.

0·00	1·00	10·07	0·00
0·20	1·00	8·42	0·165
0·40	1·00	6·81	0·326
0·60	1·00	5·24	0·483
0·80	1·00	3·64	0·643
1·00	1·00	2·08	0·799

Results approximating to the theoretical can be obtained at room temperature by a direct titration with iodine if the cysteine is contained in approximately 80 % acetic acid solution. The presence of this high concentration of acetic acid appears to minimise side-reactions and to ensure oxidation

to disulphide, but because of the low concentration of water, starch cannot be used as an indicator and the end-point must be judged by the first persistent yellow tinge imparted to the solution by excess of iodine.

Derivatives of cysteine.

The cysteine titration curve differs remarkably from that of apparently closely related thiol compounds, *e.g.* glutathione, ergothioneine, simple mercaptans and thiol acids (see below). It was hoped that by studying the reactions of compounds formed by substituting the hydrogen in the carboxyl group of cysteine, the amino-group and finally both groups, some explanation might be found for the peculiar peak at about p_H 2 in the titration curve. Several derivatives of cysteine were accordingly synthesised and their iodine consumptions were studied at different acidities and temperatures.

The first derivatives studied were those in which the carboxyl group was esterified. The ethyl ester hydrochloride of cystine was prepared by passing dry HCl gas into a suspension of cystine in warm absolute alcohol. After boiling on a water-bath for 12 hours, until the cystine had all dissolved, the solution was filtered hot and set in the ice-chest to crystallise. After being twice recrystallised from ethyl alcohol and dried in a vacuum over sulphuric acid, micro-Kjeldahl determinations gave 7·44, 7·47 and 7·68 % of N. Micro-determinations of S gave 17·43 %, of Cl 19·99 and 20·51 %[1]. (Theory for the dihydrochloride, 7·59 % N, 17·4 % S, 19·3 % Cl.)

In a similar manner the *iso*propyl ester was prepared and isolated as the dihydrochloride. After one recrystallisation micro-Kjeldahl gave 7·6 % N; theory requires 7·06 %.

These substances were reduced with zinc in aqueous hydrochloric acid solution immediately before titration, filtered and diluted to volume with oxygen-free water.

A derivative with the amino-group protected was prepared by forming acetylcysteine by a modification of Pirie's [1931] method. The product was practically free from any odour of acetic acid. Analysis, 8·36, 8·38 % N; calc. 8·52 %.

Finally, a compound with both groups substituted, the ethyl ester of acetylcysteine was prepared by acetylation of the ethyl ester of cystine in pyridine. The ethyl ester of diacetylcystine gave on analysis 7·38, 7·58 % N; 16·93, 17·33 % S; (Theory 7·37 % N; 16·84 % S). This was reduced with zinc dust in acid solution just before titration, as in the case of the simple esters.

Effect of substitution in the carboxyl group (ethyl and iso*propyl esters of cysteine).* Solutions of cysteine ethyl ester are much more stable than cysteine solutions. On standing in an open flask at room temperature for 8 days less than 20 % of the compound in solution was oxidised.

Between p_H 0 and 5·5 there was no variation in the iodine uptake. Above p_H 6 slightly larger amounts of iodine were combined. There is, however, an

[1] We are indebted to Dr Helen Stantial for the micro-analyses reported in this paper.

appreciable temperature effect, as shown in Fig. 1. The *iso*propyl ester behaves similarly.

Effect of substitution in the amino-group (acetylcysteine). The peculiar increase in the iodine consumption of cysteine on direct titration reaches its maximum at p_H 1·8, at which acidity the iodine uptake is almost 50 % greater than in a solution of p_H 0. In the case of acetylcysteine the maximum occurs at p_H 4·5 and is much more pronounced, over 100 % more iodine being required than at p_H 0 (Fig. 4).

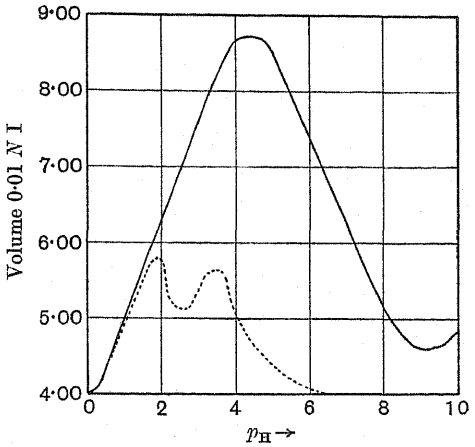


Fig. 4. Iodimetric titration of acetylcysteine at different p_H values: 0·04 millimol. acetylcysteine in 12 cc. buffer; direct titration at 0°. Dotted line is curve for cysteine titrated under similar conditions.

Effect of substitution in both the carboxyl and amino-groups (ethyl ester of acetylcysteine). In the case of the ethyl ester of acetylcysteine, the iodine consumption was practically constant between p_H 0 and 8.

From these results it would appear that an appreciable rise in the curve relating iodine consumption with p_H occurs only if the carboxyl group of cysteine remains free. Esterification eliminates the variations in iodine consumption between p_H 0 and 5, giving compounds similar in their behaviour in this respect to glutathione and to simple thiol acids. The shift of the region of maximum iodine consumption to a less acid range and the augmentation of iodine uptake in the case of acetylcysteine may possibly be related to the fact that on introducing an acyl group into the amino-group its basic properties are weakened, allowing a corresponding increase in the strength of the adjacent carboxyl group.

Glutathione.

One of the early improvements in the estimation of glutathione, based on Tunnicliffe's [1925] iodimetric procedure, was the use of excess iodine in the presence of potassium iodide by Perlzweig and Delrue [1927]. The potassium iodide stabilises the starch end-point by minimising the extent to which side-

reactions occur. Kuhnau [1931] gave a careful review of the biochemical literature on the estimation of this compound, and determined the conditions under which glutathione could be most favourably determined in liver extracts. King and Lucas [1931] showed that at temperatures of 20° and under, crystalline glutathione required theoretical amounts of iodine when titrated at acidities greater than p_H 5. Above this p_H larger amounts were used.

The effect of dilution on the capacity of glutathione for reducing iodine, both on direct and indirect titration, and the marked action of potassium iodide in minimising this dilution error are shown in Table VII. It is noted that

Table VII. *Titration of glutathione at different dilutions.*

3·155 mg. glutathione* in *N*/100 HCl titrated at 10°.

	Direct titration				Indirect
Volume cc.	0·01 *N* I used cc.	End-point correction cc.	0·01 *N* I consumed cc.	0·01 *N* I consumed in presence of KI cc	0·01 *N* I consumed cc.
1	0·85	—	0·85	—	—
5	0·88	—	0·88	—	0·83
10	0·91	0·02	0·89†	0·84 (1 % KI)	—
20	0·98	0·05	0·93	—	—
30	1·08	0·07	1·01	—	—
50	—	—	—	—	1·31
60	1·30	0·12	1·18†	—	—
100	—	—	—	—	1·47
					0·87 (1 % KI)
120	1·57	0·30	1·27	0·90 (1 % KI) 0·86 (2 % KI)	—

* The sample of glutathione used in this experiment was a non-crystalline product prepared in 1930 from yeast by the method of Pirie [1930] and was approximately 20 % in the oxidised form at the time these titrations were made.

† The 10 cc. dilution corresponds roughly to the concentration of glutathione in a 10 % extract of liver, and the 60 cc. dilution corresponds roughly to that in a 1 in 10 trichloroacetic acid filtrate of blood.

in the neighbourhood of the glutathione concentration commonly reported for liver extracts there is approximately an 8 % error in the amount determined by direct titration. In the case of the concentration corresponding approximately with those reported for blood-filtrates there is a 40 % error on direct titration and a 60 % error on indirect titration. These errors would appear to be eliminated by the presence in the solution of 1 % potassium iodide, without suppressing the iodine uptake below the point corresponding to simple oxidation to disulphide. The above titrations were made at p_H 2, but it was also found that the addition of 2 % potassium iodide diminished the iodine uptake almost to the theoretical even when the titration was carried out at neutrality.

Ergothioneine[1] (*the betaine of thiolhistidine*). On account of the occurrence of this sulphydryl compound in blood-filtrates its behaviour towards iodine under various conditions of temperature and acidity was investigated. An approximately *M*/100 solution was prepared by dissolving 33 mg. of the dry

[1] The ergothioneine used in this experiment was kindly supplied to us by Prof. G. Hunter, of the University of Alberta, to whom our thanks are due.

substance in 15 cc. of freshly boiled distilled water. At 0° 1 cc. required 0·892 cc. of 0·01 *N* I by direct titration from a micro-burette; at 20° 0·904 cc. were required, and at 40° 0·924 cc.; the temperature effect, therefore, is almost negligible. That the dilution effect is also very small would appear from the fact that 1 cc. undiluted required 0·904 cc. of 0·01 *N* I, 1 cc. diluted to 10 with distilled water required 0·935 cc. iodine; diluted to 20 cc. it required 0·920 cc. and to 50 cc. 0·890 cc.

Titration of ergothioneine at different hydrogen ion concentrations using glycine, acetate and phosphate buffers, showed that the p_H effect was small on the acid side of neutrality, being almost a straight line between p_H 0 and 6·4. At reactions more alkaline than p_H 7 very much larger amounts of iodine were consumed, and the end-point was transient. By titration of excess iodine with thiosulphate it was found that a total of six times as much iodine was required as that used below p_H 7 (see Table VIII).

Table VIII. *Effect of* p_H *on iodine titration of ergothioneine.*

1 cc. of *M*/100 ergothioneine + 4 cc. buffer titrated at 21° with 0·01 *N* I.

p_H	cc. I	p_H	cc. I
0	0·889	7·0 (first transient end-point)	0·95
1	0·900	7·0 (final end-point)	1·03
2	0·930	7·3 (first end-point)	1·2
3·7	0·945	7·3 (final end-point)	5·9
5·8	0·960	9 (first end-point)	3
6·4	0·950	9 (final end-point by back titration)	5·9
6·4 (without buffer)	0·930		

The presence of added potassium iodide in an ergothioneine solution appeared to have a most marked effect on the amount of iodine consumed. Thus 1 cc. of 0·01 *M* ergothioneine in 5 cc. distilled water required 1·03 cc. 0·01 *N* I, while the presence of 1 % KI depressed the iodine consumption to 0·76 cc. In acid solution the discrepancy was greater: 1 cc. in 5 cc. of *N*/10 HCl required 1·00 cc. 0·01 *N* I and only 0·41 cc. in the presence of 1 % KI. 1 cc. of ergothioneine in 10 cc. of 2·5 % trichloroacetic acid required 0·99 cc. I and the addition of 1 % of KI caused the amount consumed to fall to only 0·20 cc. Under the condition most favourable to titration of cysteine and of glutathione, therefore, ergothioneine reduces only a small fraction of its theoretical equivalent of iodine. This point has recently been considered by Meldrum [1932] and Woodward and Fry [1932] in their study of blood-glutathione determination.

Thiophenols.

In the case of thiophenol and thiocresol the iodine consumption was constant between p_H 0 and 7. At p_H 7·5 a definite increase was noted. The temperature effect was almost nil (see Figs. 1 and 5).

Thio-acids.

Thioglycollic acid and thiolactic acid were chosen as being examples of simple thiol compounds containing a carboxyl group. The marked influence of

temperature when the titration was carried out in N hydrochloric acid is shown in Fig. 1. At 0° the amount of iodine combined corresponded most closely with the stoichiometric relationship $2XSH + I_2 \rightarrow XSSX + 2HI$. The iodine consumption was constant between p_H 0 and 6·5, as shown in Fig. 5.

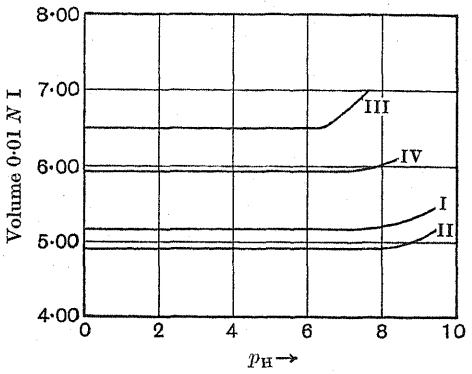


Fig. 5. Effect of p_H on the iodine consumption of thiophenols and thiol-acids. (I) 1 cc. 0·052 M thiophenol, (II) 1 cc. 0·049 M thiocresol, (III) 2 cc. 0·033 M thioglycollic acid, (IV) 2 cc. 0·03 M thiolactic acid.

Summary.

Temperature exerts a marked effect on the amount of iodine combined by cysteine in solution. Extraordinary differences are found in the amounts of iodine consumed at different hydrogen ion concentrations. Esterification of the carboxyl group lessens the temperature effect and almost eliminates the c_H effect. Acetylation of the amino-group, on the other hand, increases the maximum of iodine consumption and alters its position on the p_H curve. The concentration of cysteine in the solution titrated and the concentration of the iodine used have a marked effect on the extent to which oxidation occurs. In the presence of 0·5 % potassium iodide cysteine can be determined quantitatively by indirect iodimetric titration at 0° and N acidity.

Glutathione reduces theoretical amounts of iodine only at an acid reaction (below p_H 5) and at a temperature less than 25°. At high dilution the large error which results on either direct or indirect titration can be eliminated by the presence of potassium iodide in the glutathione solution.

Cysteine in its reactions with iodine is much more markedly affected by temperature, c_H and dilution than is the tripeptide, glutathione, of which it is a constituent.

The dissimilarity of the p_H-iodine consumption curve for liver extract from that for cysteine appears to indicate the absence of appreciable amounts of free cysteine from liver.

The iodine consumption of ergothioneine appears to be only slightly affected by temperature, c_H below neutrality and dilution. The presence of added potassium iodide markedly diminishes the iodine consumption, particularly at acid reactions.

Simple mercaptans may be quantitatively determined by direct titration at room temperature. On the acid side of neutrality the acidity of the medium appears to have no effect on the iodine uptake.

The iodine consumption of thiol acids is markedly affected by temperature, theoretical iodine consumption only occurring at or near 0°. The influence of c_H is not apparent on the acid side of neutrality.

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CCXLVIII. THE PREPARATION OF METHYL ESTERS OF PECTIC ACID.

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It has been generally accepted that the naturally occurring water-soluble pectins are methyl esters of pectic acid, this latter substance, which is insoluble in water, being regarded as containing 4 galacturonic anhydride units, whose carboxyl groups are intact and are involved in the formation of the methyl esters. This conception has been based on the fact that the soluble pectins, on treatment with very dilute alkali in the cold, are rapidly converted into free pectic acid, with liberation of methyl alcohol. Further, the methoxyl-content of samples of soluble pectin prepared from fruits, *etc.* frequently corresponds closely with that required for a tri- or tetra-methyl ester of pectic acid [*cf.* Norris, 1926; Norman, 1928]. So far as can be ascertained, however, no attempt has been made to confirm this view by converting pectic acid into its methyl esters. The accomplishment of this reaction is necessary before the relation between pectin and pectic acid can be regarded as firmly established, for observations have been reported from time to time which suggest that the relationship may not be so simple. For instance, Tutin [1921; 1923] reported the liberation of acetone during treatment of pectin by weak alkali, while Ehrlich and Schubert [1926] consider that acetyl groups are also present.

Experiments have accordingly been made in an attempt to prepare the methyl esters of pectic acid; soluble methyl esters have been obtained and have been found to resemble the natural soluble pectins in all respects. There seems therefore no reason to suggest that any reaction other than hydrolysis of the methyl ester is involved in the formation of pectic acid from pectin.

Similar attempts to prepare other (*e.g.* ethyl) esters of pectic acid were, however, entirely unsuccessful. It appears, in fact, that the methyl derivatives are possibly the only stable esters of pectic acid, and that, if other esters are formed, they undergo ready decomposition, not only with loss of the ester group, but actually with scission of the basal pectic molecule.

Experimental.

Preparation of the methyl esters.

Since the object of the experiments was to methylate only the free carboxyl groups of the pectic acid, and not the hydroxyl groups present in the uronic anhydride residues, it was obviously impossible to adopt any of the methods used in the exhaustive methylation of sugars. The following methods suggested themselves:

(*a*) treatment of calcium pectate with methyl oxalate;
(*b*) treatment of silver pectate with methyl iodide;
(*c*) treatment of sodium pectate with methyl sulphate.

For these experiments, calcium pectate was prepared by precipitating a solution of pectin, after treatment for 4 hours with dilute sodium hydroxide, with calcium chloride solution in the presence of acetic acid. Sodium pectate was prepared by the method of Candlin and Schryver [1928] from the same sample of commercial pectin used by these workers. Silver pectate was prepared by addition of aqueous silver nitrate to a 1 % solution of sodium pectate; the silver compound was precipitated as a clear gel, which was thoroughly washed with cold water, and then with graded strengths of alcohol, and finally dried *in vacuo*. The washing and drying were carried out in weak artificial light, since the compound was found to darken rapidly in daylight. The dry silver pectate was obtained as a cream-coloured powder; the Ag content of different samples varied from 30·3 to 32·1 %, the highest figure being obtained in the case of a sample in the preparation of which a large excess of silver nitrate had been used. Calculated on the basis of the Nanji, Paton and Ling [1925] formula for pectic acid, the Ag content of silver pectate should be 30·3 %. The purity of the samples of calcium and sodium pectate was also checked by ash determinations.

Method (*a*). 1 g. of powdered calcium pectate, suspended in 25 cc. dry methyl alcohol, was heated with 5 g. methyl oxalate for 3 hours in a pressure bottle immersed in boiling water. At the end of this time, the mixture was added to 200 cc. hot water, filtered and the filtrate poured into 500 cc. alcohol. A small gelatinous precipitate was obtained, which, after washing with dilute alcohol and drying, weighed 0·09 g. Its methoxyl content, determined by micro-Zeisel, was 3·4 %. It seemed therefore that some esterification had taken place, but the poor yield and low methoxyl content of the product led to the abandonment of the method.

Method (*b*). A number of attempts were made to prepare the methyl esters by the action of methyl iodide on silver pectate under different conditions; the results are summarised in Table I. Usually, 1–2 g. of dry silver pectate were heated with 10–15 g. methyl iodide in 25 cc. methyl alcohol; at the end of the period of heating, the mixture was added to about 300 cc. water, boiled for a short time, the residue of silver iodide and unchanged silver pectate filtered or centrifuged off and the filtrate treated with excess of alcohol to

precipitate the soluble pectins formed. Considerable difficulty was experienced in removing colloidal silver iodide, the presence of which rendered filtration extremely slow and resulted in contamination of the alcohol precipitate with as much as 25 % of inorganic matter. The most satisfactory method of removing this colloidal material was by adding a few cc. of 5 % potassium bromide and allowing the liquid to stand on the steam-bath for an hour. The silver iodide was thus coagulated; from the clear filtrate, the pectic substance was precipitated as a transparent gel which, when dry, contained only about 3 % ash in most cases.

Table I.

Exp. No.	Treatment of silver pectate	Yield of sol. pectin (ash-free) per g. silver pectate g.	Ash in soluble pectin %	Methoxyl content of sol. pectin (corr. ash) %
1	Boiled with CH_3I under reflux, 8 hours	0·1	—	<3
2	Heated with CH_3I at 96° in pressure bottle, 1 hour	0·35	13*	4·2
3	Heated as above, 4½ hours	0·30	3·5	7·28
4*a*	Heated as above, 12 hours	—	2·3	10·50
4*b*	,,	0·42	6·0	8·60
5	Heated in sealed tube at 100°, 12 hours	—	2·3	5·95
6	Heated as above, 48 hours	—	—	6·0

* Silver iodide incompletely coagulated; boiled with $N/75$ HCl in place of potassium bromide.

From the results recorded above, it appeared that the greatest degree of esterification was produced by heating silver pectate with methyl iodide and methyl alcohol under pressure at 96° for several hours; heating at slightly higher temperatures, or for prolonged periods, resulted in much charring. The products obtained resembled in appearance and solubility the natural soluble pectins; further tests of their identity are described below.

Since a rather wide variation in the methoxyl content of the various products was met with, it was of interest to ascertain whether this was due to the fact that only partial esterification of the carboxyl groups had taken place, or whether substances other than pectins had been formed and carried down in the alcohol precipitate along with the pectins. Accordingly the calcium pectate values for certain of the products were determined; from these a calculation was made of the true methoxyl content of the actual pectic substance present [*cf.* Norman, 1928]. The results (Table II) suggested that even when conditions were apparently most satisfactory there was some production of non-pectin degradation products, possibly hemicellulose-like bodies. As will be seen later, these may in some cases comprise the major part of the products. It may be pointed out that in the case of sample 2, the apparent 25 % of non-pectic material includes the 13 % ash present in the sample; hence the actual amount of organic contaminant is only about 10 %.

Table II.

Sample No.	Methoxyl-content %	Ca pectate value %	MeO, corr. by Ca pectate %
2	3·65	75	4·85
4*a*	10·26	91	11·51
4*b*	8·09	81	9·98
5	5·82	68	8·55

In column 2, the methoxyl content of the sample, uncorrected for ash, is recorded, since the correction applied on the basis of the calcium pectate value will cover this.

Since this sample was prepared by heating only for 1 hour, the low methoxyl value (4·85 %) indicated incomplete esterification, and not extensive decomposition. In the case of sample 5, the prolonged heating of the reaction mixture led to the formation of large amounts of degradation products, reflected in the low calcium pectate value.

With regard to the yield of soluble pectin, in Exp. 4*b* the amount of true pectic substance obtained from 1 g. silver pectate was 0·35 g.; since the theoretical yield of tetramethyl ester per g. of silver pectate is 0·74 g., this represented a yield of approximately 47 %. The solid residue in the pressure bottle was found to contain 0·18 g. unchanged silver pectate.

Method (*c*). A number of attempts to convert sodium pectate into soluble methyl esters by the action of dry methyl sulphate proved entirely unsuccessful. When the substances were heated together at 60–70°, with precautions to exclude moisture, slight darkening of the sodium pectate took place, but on examining the residue (after washing away the methyl sulphate with alcohol), this was found to consist of unchanged sodium pectate. In the presence of moisture, pectic acid was formed from the sodium salt, and this was further attacked by the methyl sulphate (or by sulphuric acid produced by partial hydrolysis thereof) with liberation of carbon dioxide and complete breakdown of the pectin molecule. In no case could any substance resembling pectin be isolated from the mixtures.

Properties of the methyl esters obtained.

As stated above, the appearance and solubilities of the products obtained by the silver pectate-methyl iodide reaction resembled those of the natural soluble pectins. Treatment with lime water produced an immediate precipitate of a typical calcium pectate gel. In the presence of $N/10$ sodium hydroxide, methyl alcohol was rapidly liberated, de-esterification being complete within 15 minutes—a rate comparable with that observed in the case of natural soluble pectin. The soluble pectins were strongly dextrorotatory.

A marked characteristic of the natural soluble pectins of high methoxyl content is their ability to form jellies with sucrose in the presence of traces of acid; among the pectic substances, soluble pectin alone possesses this property [Branfoot, 1929, p. 128]. On this account, sucrose-acid jellies were made from the synthetic methyl esters, and their strengths compared with those

of similar jellies prepared from natural pectin. Jelly strengths were estimated in an apparatus similar to the "pectinometer" of Luers and Lochmüller [1927], but adapted to deal with quantities of 10 cc. of jelly, since the amounts of soluble pectin available would not permit of the preparation of larger quantities. The results are shown in Table III (methoxyl contents shown are corrected on the basis of the calcium pectate value of the sample).

Table III.

Sample	Methoxyl-content %	Jelly strength: 1·5 % pectin g.	Jelly strength: 0·75 % pectin g.
Grape-fruit pectin	11·60	320	—
Commercial citrus pectin*	9·1	270	175
Methyl ester No. 4*a*	11·51	310	180
Methyl ester No. 4*b*	9·98	285	—
Methyl ester No. 5	8·55	180	105

* Pectin from which sodium pectate and silver pectate used in these experiments was prepared.

The results indicated quite clearly that the esters now prepared were comparable in jellying properties with the original natural soluble pectins.

Examination of the figures for methoxyl content of the various products suggested that in each case mixtures of the esters were formed, since none of the figures approximated to the methoxyl-content of a definite di-, tri- or tetra-methyl ester of pectic acid.

Attempted preparation of ethyl esters.

So far as is known, only the methyl esters of pectic acid occur naturally [von Fellenberg, 1918]. In order to see whether other esters resembled the methyl derivatives in their properties, an attempt was made to prepare the ethyl esters by heating silver pectate and ethyl iodide together under the same conditions as were found successful in producing methylation.

During the reaction, the mixture was observed to become very dark in colour. The aqueous extract after the reaction gave no precipitate with alcohol, but on evaporation left a brownish gum, which gave a strong reduction with Fehling's solution. Since an examination of the solid residue in the pressure bottle showed this to consist almost entirely of silver iodide, with a little unchanged silver pectate, it seemed likely that the reaction between the silver pectate and ethyl iodide had actually taken place with formation of ethyl pectates, and that it was the esters which underwent subsequent decomposition, with formation of the gummy reducing substances. This view was further supported by the observation that pectic acid itself was not appreciably destroyed by heating for some hours at 96° with ethyl iodide, alone or in the presence of absolute alcohol. It may be suggested, tentatively, that the introduction of a heavier group into the pectic molecule (even the substitution of C_2H_5— for CH_3—) results in an instability of the pectic ring, and its consequent breakdown to simpler substances. In this connection one may recall

the comparative ease with which pectins have been found to break down into hemicellulose-like bodies, sugars, *etc.* [*cf.* Candlin and Schryver, 1928; Linggood, 1930; Norman and Norris, 1930].

Summary.

1. Pectic acid is readily converted into its methyl esters by heating silver pectate with methyl iodide under slight pressure. The products resemble the natural soluble pectins in all respects.

2. There is no reason to suppose that the soluble pectins are other than simple methyl esters of pectic acid.

3. The ethyl esters of pectic acid are apparently unstable, undergoing extensive decomposition under the experimental conditions used in their preparation.

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APPENDIX.

Small-scale "pectinometer."

The small-scale "pectinometer" used in testing the strengths of the sugar-pectin jellies in the above work was devised and made by our laboratory assistant, Mr G. Howkins. It was designed to deal with one-tenth the amount of jelly used in the ordinary pectinometer, *i.e.* 0·15 g. pectin in 10 g. jelly.

The vessel in which the jelly is allowed to set consists of a piece of thin copper tubing, 6·5 cm. long and 2·4 cm. internal diameter closed at the bottom. Into the sides of this are drilled and soldered 8 pieces of brass wire (S.W.G. 16), projecting 5 mm. into the tube; these are arranged in two circles, the lower being 1 cm. and the upper 1·5 cm. from the bottom of the tube, and serve to hold the jelly firmly in the tube. Along the axis of the tube is fixed a piece of similar wire,

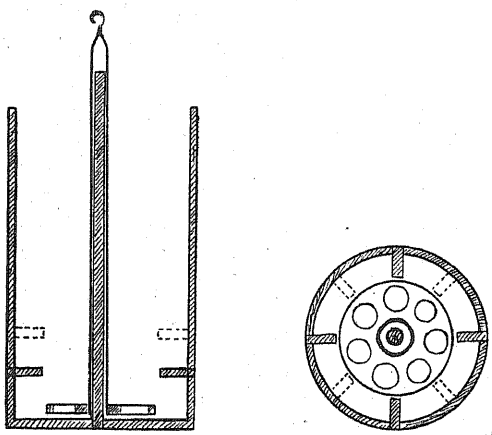


Fig. 1. Small-scale "pectinometer"; arrangement of vessel in which jelly is "set," and device for "tearing" jelly.

8 cm. long, to serve as a guide for the jelly-tearing device. This latter consists of a thin copper tube, 10 cm. long and as narrow as possible, which just slides easily over the guide-wire; to its lower end is fixed a circle of brass, 12 mm. diameter, perforated with 7 holes at its periphery. The upper end of the inner tube carries a hook, to which is attached a fine chain, passing over two pulley-wheels to a counterpoised balance-pan, as in the ordinary pectinometer.

In making the test, sufficient of the pectin sample to yield 0·15 g. calcium pectate is dissolved in 10 cc. of 0·2 % tartaric acid solution, together with 6 g. sucrose. The mixture is boiled in a 30 cc. beaker until the weight of the mixture is reduced to 10 g. (By means of a preliminary test, a mark is made on the beaker to indicate this point.) The boiling is completed within 15 minutes. The hot mixture is transferred immediately to the setting-tube of the pectinometer, in which it forms a layer about 2 cm. deep. The subsequent procedure in estimating the strength of the jelly is exactly the same as in the case of the ordinary pectinometer. The apparatus has proved sufficiently satisfactory, giving comparable results within about 5 %.

CCXLIX. THE RAT UNIT OF TESTICULAR HORMONE.

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In previous papers [Korenchevsky, 1932, 1, 2; Korenchevsky, Dennison and Schalit, 1932] a detailed description has been given of the method of assay of testicular hormone, using rats as the experimental animals, and a new rat unit was suggested for use in the assay of this hormone.

In the present investigation a test was made of the method of assay and the suitability of this rat unit, and a comparison of the size of the rat unit (RU) with that of the capon unit (CU).

Technique.

Experiments were performed on 42 rats belonging to 8 litters. The technique of the assay, described previously, was followed strictly. The ages of the rats at castration, the number of days after castration before the injections were started, and the weights of the rats at the end of the experiments are given in Table I.

Table I. *Age and average final weights of the rats in each group of each litter.*

No. of litter	No. of rats in litter	Age at castration (days)	No. of days after castration injections started	Weight of rats (g.)					
				Control rats	Rats injected with				
					1·0 CU	1·5 CU	2·5 CU	5·0 CU	7·5 CU
1	6	24	50	221	230	234	—	—	—
2	7	24	50	197	182	204	—	—	—
3	5	27	43	166	184	182	—	—	—
4	3	29	32	174	—	—	193	—	—
5	6	27	32	217	—	—	—	218	210
6	5	27	32	194	—	—	209	—	—
7	5	22	32	192	—	—	—	191	—
8	5	25	75	343	—	—	—	387	—

A well-purified testicular hormone, isolated from urine, belonging to one and the same batch, prepared by Messrs Schering Ltd., was used for the injections[1]. The hormone was dissolved in oil and its strength was stated to

[1] We wish to express our thanks to Messrs Schering Ltd. for kindly supplying this preparation.

be equal to 25 capon units per cc., as standardised in the Schering Laboratories. We have not re-tested the preparation on capons, and in the following its strength will be designated in capon units according to Schering's standardisation.

For the injection of small doses, sufficient of the original preparation for the day's injection was diluted, each morning, with sesame oil under aseptic precautions. For the injection of 5·0 and 7·5 CU daily the undiluted original preparation was used. The daily dose was injected subcutaneously twice a day, in half doses, at about 10 a.m. and 5 p.m. for 7 consecutive days.

After the morning injection the remaining doses for the evening injection were covered with sterilised glass lids and placed in the cold store. In no case were indurations or any local reactions noticed, nor was there depression of the animals or of their appetite.

All the litters were castrated before sexual maturity at the age of 22 to 29 days.

As can be seen from Tables II to VI, only the first 7 litters were used in the standard test. These were chosen intentionally so that the number of days after castration was between 32 and 50 days and therefore the weights of the rats did not differ greatly—the average weights at the end of the experiment being between 166 and 234 g.

Litter 8, which was about double the weight of the other litters (see Table I), was chosen to show the correlation between the dose and the weight of the rat. The results of the injections into the rats of this litter are discussed below in a separate paragraph and are not included in Tables II to VI in order to avoid confusion. The supply of testicular preparation was unfortunately insufficient for the treatment of more than one litter of large rats.

The influence on prostate with seminal vesicles.

The results obtained are summarised in Table II. In previous work [Korenchevsky *et al.*, 1932] experiments showed that the variations in the increase of prostate with seminal vesicles were not large in rats belonging to different litters injected at a similar period after castration with the same dose of the same testicular extract, provided the rats were operated on at the same time in relation to sexual maturity. The same results were obtained in the present experiments, as can be seen from a comparison of the percentage increase of prostate with seminal vesicles of rats injected with the same dose but belonging to different litters (Table II). The variation was considerable in only one group of the rats injected with one CU. However, it must be remembered that this, the smallest dose of one unit, would give an increase comparatively close to the limit of physiological variations [Korenchevsky, 1932, 2] of the weight of prostate with seminal vesicles. In a standardisation using the smallest dose, therefore, it is sometimes difficult in a biological test to rely on an increase of 20 or 30 mg. in the weight of the organs, since physiological variation of a few mg. will influence the results obtained considerably.

Table II. *Influence on prostate with seminal vesicles.*

The average weights, actual and calculated, per 200 g. of body weight, of the prostate and seminal vesicles of the rats in each group of each litter and their percentage increase after the injection of testicular hormone.

No. of litter	Control rats	Rats injected with					Percentage increase in weight				
		1 cu	1·5 cu	2·5 cu	5·0 cu	7·5 cu	1·0 cu	1·5 cu	2·5 cu	5·0 cu	7·5 cu
					A. Actual weights.						
1	64	97	105	—	—	—	51·5	64·0	—	—	—
2	62	81	96	—	—	—	30·6	54·8	—	—	—
3	71	100	117	—	—	—	40·8	64·8	—	—	—
4	56	—	—	120	—	—	—	—	114·1	—	—
5	55	—	—	—	160	219	—	—	—	190·8	298·0
6	56	—	—	111	—	—	—	—	98·2	—	—
7	53	—	—	—	154	—	—	—	—	190·6	—
Av.	**60**	**93**	**106**	**116**	**157**	**219**	**41·0**	**61·2**	**106·2**	**190·7**	**298·0**
					B. Weight calculated per 200 g. body weight.						
1	58	85	90	—	—	—	46·5	55·2	—	—	—
2	63	90	94	—	—	—	42·8	49·2	—	—	—
3	87	109	129	—	—	—	25·3	48·3	—	—	—
4	64	—	—	124	—	—	—	—	93·8	—	—
5	51	—	—	—	146	212	—	—	—	186·2	315·5
6	58	—	—	106	—	—	—	—	82·9	—	—
7	55	—	—	—	162	—	—	—	—	194·5	—
Av.	**62**	**95**	**104**	**115**	**154**	**212**	**38·2**	**50·9**	**88·4**	**190·4**	**315·5**

To correct this at least 3 litters should be used and the averages can then be considered reliable and, as can be seen from the tables, this procedure gives satisfactory results. It would be still better to use for the standardisation doses of about 2 or 3 units when small physiological variations would be relatively less important.

In the previous experiments a close correlation of direct proportion was obtained between the increase in the dose of the hormone injected and the average increase of the prostate with seminal vesicles.

It can be seen from Tables II and V that the same correlation was found in the present experiments. At the same time, when the weights of the rats are so nearly the same as was the case in our experiments, the average percentage changes for the actual weights are very similar to those calculated for the same unit of rat's weight. In both our previous and our present experiments the unit of 200 g. of body weight was chosen, since this is the average weight of the rats most often used for the experiments.

The influence on the penis.

As was stated in the previous paper, it was found that the penis responds to the injection of testicular hormone more slowly and in a less regular way than the prostate with seminal vesicles. However, as is shown in Tables III and V, the present experiment corroborates our previous statement, that, for the assay, the changes in the penis give an extra proof of the activity and strength of the preparation tested.

Table III. *Influence on the penis.*

The average weights in mg., actual and calculated per 200 g. of body weight, of the penis of the rats in each group of each litter and their percentage increase after the injection of testicular hormone.

No. of litter	Control rats	Rats injected with					Percentage increase in weight				
		1·0 CU	1·5 CU	2·5 CU	5·0 CU	7·5 CU	1·0 CU	1·5 CU	2·5 CU	5·0 CU	7·5 CU
					A. Actual weights.						
1	82	97	98	—	—	—	18·3	19·5	—	—	—
2	67	79	80	—	—	—	17·9	19·4	—	—	—
3	67	80	103	—	—	—	19·4	53·7	—	—	—
4	67	—	—	104	—	—	—	—	55·2	—	—
5	64	—	—	—	110	137	—	—	—	71·9	114·0
6	70	—	—	111	—	—	—	—	58·6	—	—
7	66	—	—	—	110	—	—	—	—	66·6	—
Av.	**69**	**85**	**94**	**108**	**110**	**137**	**18·5**	**30·9**	**56·9**	**69·3**	**114·0**
					B. Weights calculated per 200 g. body weight.						
1	74	85	84	—	—	—	14·8	13·5	—	—	—
2	68	87	78	—	—	—	27·9	14·7	—	—	—
3	82	87	113	—	—	—	6·1	37·8	—	—	—
4	77	—	—	108	—	—	—	—	40·3	—	—
5	59	—	—	—	100	131	—	—	—	69·5	122·0
6	73	—	—	100	—	—	—	—	37·0	—	—
7	69	—	—	—	115	—	—	—	—	66·7	—
Av.	**72**	**86**	**92**	**104**	**108**	**131**	**16·3**	**22·0**	**38·7**	**68·1**	**122·0**

Table IV. *Influence on the thymus.*

The average weights of the thymus (mg.) calculated per 200 g. body weight of the rats in each group of each litter and their percentage decrease after the injection of testicular hormone.

No. of litter	Control rats	Rats injected with					Percentage decrease in weight				
		1·0 CU	1·5 CU	2·5 CU	5·0 CU	7·5 CU	1·0 CU	1·5 CU	2·5 CU	5·0 CU	7·5 CU
1	508	398	410	—	—	—	21·6	19·3	—	—	—
2	644	508	529	—	—	—	21·1	17·9	—	—	—
3	569	564	525	—	—	—	0·9	7·7	—	—	—
4	639	—	—	611	—	—	—	—	4·4	—	—
5	850	—	—	—	616	607	—	—	—	27·5	28·6
6	702	—	—	666	—	—	—	—	5·1	—	—
7	862	—	—	—	734	—	—	—	—	14·8	—
Av.	**682**	**490**	**488**	**639**	**675**	**607**	**14·5**	**15·0**	**4·8**	**21·2**	**28·6**

In the most recent paper [Korenchevsky, 1932, 2] it was stated that the weighing of the dissected penis is more accurate if it is fixed in Bouin's fixative before weighing (*i.e.* the penis should be treated in the same way as the other organs). In this way more regular changes after injection were obtained in the penis.

From Tables III and V it is clear that, although the response of the penis to the injections was less sensitive, this organ, like the prostate with seminal vesicles, showed on the average changes in weight in direct proportion to the dose injected; for each RU or CU an increase was obtained in the weight of the

Table V. *The relation between the rat unit and the capon unit of testicular hormone.*

Dose of CU injected	Average % increase in weight: Obtained	Expected	% increase obtained per CU: With each dose	Average (max. % deviations in brackets)	No. of rat units in dose
Prostate + seminal vesicles:		A. Actual weights.			
1·0	41·0	—	41·0		1·03
1·5	61·2	61·5	40·8	40·4	1·53
2·5	106·2	102·5	42·5	(+ 5·2 − 5·7)	2·66
5·0	190·7	205·0	38·1		4·77
7·5	298·0	307·5	39·7		7·45
Penis:					
1·0	18·5	—	18·5		—
1·5	30·9	27·8	20·6	18·2	—
2·5	56·9	46·3	22·8	(+25·3 −23·6)	—
5·0	69·3	92·5	13·9		—
7·5	114·0	138·8	15·2		—
		B. Weights calculated per 200 g. body weight.			
Prostate + seminal vesicles:					
1·0	38·2	—	38·2		0·96
1·5	50·9	57·3	33·9	37·5	1·27
2·5	88·4	95·5	35·4	(+12·3 − 9·6)	2·21
5·0	190·4	191·0	38·1		4·76
7·5	315·5	286·5	42·1		7·89
Penis:					
1·0	16·3	—	16·3		—
1·5	22·0	24·5	14·7	15·3	—
2·5	38·7	40·8	15·5	(+ 6·5 −11·1)	—
5·0	68·1	81·5	13·6		—
7·5	122·0	122·3	16·3		—

penis of about 18 % (actual weight) or 15 % (when calculated per 200 g. of body weight) as compared with that of the controls.

We would, therefore, advise the use of the changes in the penis as a rough extra proof of the activity and strength of the hormone. In the case of the penis the weights calculated per 200 g. of body weight gave closer results than the changes in the actual weights (Table V).

Influence on the thymus.

The changes observed in the thymus after the injection of testicular hormone are given in Table IV. Since these changes were small, to economise space only the weights calculated per 200 g. of rat's weight are given.

The decrease in the thymus after the injection of the extract used was in most cases small, and in one case (litter 3, dose 1 CU) even negligible; also the correlation of direct proportion between the increase in the dose injected and the degree of decrease is not clearly shown. In the previous experiments similar changes were noted. Nevertheless, in spite of the unsuitability of these changes for standardising the hormone, we would suggest that they be noted during an assay of a preparation as indicating the activity of that preparation on the thymus.

The influence on fat deposition, adrenals, thyroids and hypophysis.

As was found in previous experiments, seven days of injection is too short a period for the production of marked changes in these organs and in fat deposition. For this reason only the average figures of the results obtained with each dose are given (Table VI).

Table VI. *Average percentage changes in weight (actual and per 200 g. of rat's weight) of retroperitoneal fat, adrenals, thyroid and hypophysis of the injected rats compared with those of the control rats.*

	Rats injected with				
Organs	1·0 CU	1·5 CU	2·5 CU	5·0 CU	7·5 CU
Retroperitoneal fat:					
Actual weight	+37·4	+37·0	+13·3	−14·6	−15·9
Per 200 g. rats weight	+34·2	+39·0	+ 6·5	−14·5	−12·4
Adrenals:					
Actual weight	− 1·1	+ 2·1	− 8·2	− 9·4	−16·7
Per 200 g. rats weight	− 4·3	− 5·0	−16·0	−10·0	−13·2
Thyroid:					
Actual weight	+ 7·6	+ 7·6	+ 6·3	+ 9·0	+10·9
Per 200 g. rats weight	+ 4·1	+ 0·3	− 1·5	+ 8·5	+13·4
Hypophysis:					
Actual weight	− 1·2	− 5·2	− 9·4	+ 2·8	− 8·5
Per 200 g. rats weight	− 2·6	−11·2	−19·4	+ 1·1	− 6·8

It has already been stated [Korenchevsky, 1932, 1, p. 420] that, in order to be certain that the changes found after injection are outside the limits of physiological fluctuations, the increase in weight of the thyroids should be not less than 40 %, while for the decrease in weight of the adrenals, hypophysis and fat deposition an even larger figure should be allowed. The changes obtained in the present experiment are therefore too small to permit of any definite conclusions. We would like to note, however, that, though small, there was, in most cases, an increase in the weights of the thyroids and a decrease in the weights of the adrenals and hypophysis, that is, there was a tendency for the organs changed by castration to return to normal.

One more point must be mentioned here, that in castrated rats a period of 1 or 2 months after castration is not sufficient to produce maximum changes in the above organs [Korenchevsky, 1931, 2]. It is therefore necessary, in testing the influence of testicular hormone on these organs, to choose rats which have been castrated not less than 3 months, preferably more, before the test. For the same reasons the changes in fat deposition were also small, while the physiological variations in fat deposition were greater than in any of the organs which have been mentioned [Korenchevsky, 1932, 1]. However, it is noteworthy that, with small doses (1·0 or 1·5 CU) fat deposition increased and with large doses (5 or 7·5 CU) decreased. The intermediate dose of 2·5 CU produced a small increase in fat deposition. If these results are not accidental, they may indicate that metabolism is differently influenced by small and by large doses of testicular hormone.

The rat unit and its relation to the capon unit.

It must be emphasised that, since we did not re-test the capon unit of the preparation used, this unit was taken to be as was stated by Messrs Schering. Up to the present time the method of assay of testicular hormone by using capons has not been standardised and therefore the capon unit may vary in different laboratories.

With reference to the disadvantages of the capon unit, which have already been discussed [Korenchevsky, 1932, 1, p. 413], it is necessary to add that Womack and Koch [1932, p. 275] have established that the urine of pregnant and of normal women yields "the same amount of comb growth-stimulating material as urine from normal mature men." Kabak [1931] arrived at a similar conclusion. Womack and Koch (p. 276) state that "we have present in urine from pregnant and normal mature women, a substance very similar to or identical with the testicular hormone[1] but that it is different chemically in some respects and acts differently physiologically from the female sex hormone which produces oestrus in the castrated female. The actual source and function of this hormone is at present a mystery." It is important that the authors failed to obtain this hormone from the urine of an ovariectomised woman, which would indicate that the ovaries are the source of this hormone. The specificity of the comb test, therefore, becomes doubtful in several instances and in any case it seems that for the assay of testicular hormone it should be used simultaneously with, if not replaced by, a test on the sexual and other organs of castrated mammals. Taking this into consideration it is necessary to discuss the results of our present experiments with reference to the suitability of the method of assay and of the rat unit as these have been described in our previous and present papers.

In order to answer this question, the results are presented in Table V so as to show the relation between the capon and rat units and the suitability of the latter. It is clear, from the second and third columns of this table, that the increase in weight of the prostate with seminal vesicles obtained in the experiment is very close to that theoretically expected, since there is a direct relationship between the increase in the amount of hormone injected and the increase in the weights of the prostate with seminal vesicles. The fourth and fifth columns show that the percentage deviations from the average increase, corresponding to 1 CU, were not more than 6 % for the actual figures or 12 % when calculated per 200 g. of rat's weight.

This may be considered quite satisfactory for a biological test. The figures in the sixth column of the table were obtained by dividing "% increase obtained" (2nd column) by 40, since the suggestion for the rat unit was the increase in weight of the prostate with seminal vesicles by 40 % as compared with those of the uninjected control rats. As can be seen from the table, the rat units were nearly equal to the capon units. They are more nearly so if we

[1] As far as can be understood from the paper the only test made was that of comb growth in capons.

take the average figure: thus the sum of the doses in CU (column 1) is 17·50 CU, with which corresponds (column 6) 17·44 RU obtained by changes in the actual weight of the prostate with seminal vesicles, or 17·09 RU, obtained by the changes per 200 g. of body weight. From this, in the batch tested, 1 CU corresponds to 0·997 RU (actual weights) or 0·977 RU (per 200 g. of body weight).

Therefore, using our method of assay and definition of the rat unit, this latter proved unexpectedly to be nearly equal to the capon unit (as assayed in the Schering Laboratory).

Precautions necessary in assaying the rat unit.

1. All the precautions and rules detailed in the previous papers are confirmed.

2. *The influence of the difference in weight of the rats.* Since the rats must be castrated before the 30th day after birth, and since the weight of the prostate with seminal vesicles remains constant (per unit of body weight) from the end of the first month after castration [Korenchevsky, 1932, 2], the rats seem most suitable for the assay at the age of about 2 months. A longer period is inconvenient since it means waiting longer until the animals are ready for the assay. In our present experiments we used, without appreciable change in the results, rats castrated 32 to 50 days before the experiments were started. This margin of about 18 days is convenient since even in large Institutions, having their own breeding rooms, it is difficult to obtain at short notice the necessary number of litters of a special age.

The amount of the testicular hormone preparation available was unfortunately only sufficient for the performance of one experiment (on litter No. 8) for studying the influence of the same dose of hormone on rats weighing about twice as much as litters 5 and 7. The weights of the rats of these litters and their ages are given in Table I. The daily dose injected was 5 CU. The results on litter 8 are given in Table VII and, as can be clearly seen, the increase in weight of the prostate with seminal vesicles was considerably less than that in litters 5 and 7 injected at the same time with the same dose, but more than in litters 4 and 6 injected with half the dose (2·5 CU).

This indicates that:

(1) if the same dose of the hormone is injected into rats of different weights, the influence will be less in heavier rats;

(2) a strict direct proportion does not exist between the weight of the rat and the effect of the dose injected per unit of body weight, for in this case the increase of prostate with seminal vesicles instead of 154 % (actual weight) and 123 % (per 200 g. body weight) would be equal to that of the rats injected with 2·5 CU, *i.e.* about 106 % and 88 % respectively, since the weight of litter 8 was about twice that of litters 4 and 6.

Since, however, the experiment was performed on only one litter more experiments must be made before drawing a definite conclusion.

Table VII. *Influence of injections of 5 CU per day on average weight of organs of rats of litter 8 as compared with that of non-injected litter-mates.*

	Prostate + seminal vesicles		Penis		Thymus		Retroperitoneal fat	
	Actual mg.	Per 200 g. body weight mg.	Actual mg.	Per 200 g. body weight mg.	Actual mg.	Per 200 g. body weight mg.	Actual g.	Per 200 g. body weight g.
Control rats	87	51	88	51	480	278	17·92	10·51
Injected rats	221	114	157	81	484	250	18·28	9·46
% change in weight of organs	+154	+123	+ 78·4	+58·8	+ 0·8	− 10·1	+2·0	−10·0

	Adrenals		Thyroid		Hypophysis	
	Actual mg.	Per 200 g. body weight mg.	Actual mg.	Per 200 g. body weight mg.	Actual mg.	Per 200 g. body weight mg.
Control rats	66·3	38·5	28·1	16·4	11·5	6·7
Injected rats	63·8	32·9	35·8	18·6	14·7	7·6
% change in weight of organs	− 3·8	−14·5	+27·4	+13·4	+27·8	+13·4

3. The dissection of the very atrophied prostate and seminal vesicles from the surrounding fat tissue is a rather delicate task and needs some training. It must be performed with the utmost accuracy, using, if necessary, a dissecting lens, and since these organs in castrated animals weigh only from 50 to 70 mg. the error in leaving a little fat tissue or removing a little of the prostatic tissue might influence the results considerably. At the same time accuracy must not interfere with rapid dissection as the organs must not be allowed to dry in the air, thus creating another source of error.

The accurate removal of fat tissue from all organs is roughly indicated by their sinking at once to the bottom of the vessel containing the Bouin's fixative. If any organ floats it must be removed from the fixative and the fat tissue left unnoticed removed.

For dissecting the hypophysis a very sharp small scalpel should be used.

4. The hormone is usually dissolved in oil and during injection it is necessary to push the needle into the subcutaneous tissue as far as possible from the point of entrance into the skin by pressing the bulb of the needle forward against the skin. In rats, if this is not done, about 15 to 30 minutes after the injection the oil begins to leak out and the loss in this way might be considerable. As a routine our rats are inspected about 45 minutes after the injections and any leakage is noted in the records.

5. The experience of Institutes breeding their own stocks of rats has shown that even with the best care it is not possible to prevent the occurrence of single cases of pneumonia among the rats. Rats suffering from pneumonia, especially those failing to grow or losing weight, must be discarded or the

results obtained with these rats must be taken with reservation, since pneumonia interferes with the influence of the injection of testicular extract. At *post mortem*, therefore, the lungs of the rats must always be examined for pneumonia.

Disadvantages of the method.

For each assay new animals must be operated on and chosen with reference to their age at castration and at the beginning of the experiment.

Advantages of the method.

1. The method of assay is accurate, precise in end-point reading, quick and simple, since it only requires the weighing of the organs and not a microscopic examination.

2. The data obtained give a direct indication of the changes in the secondary sexual organs of the male, *i.e.* the method is specific for the male hormone.

3. The method, when used for longer periods, gives an indication of the changes in most of the other organs and functions which are influenced by castration and usually, even after only 7 days, those in the thymus.

4. In order to determine the number of rat units contained in the dose injected at least 3 litters, preferably more, are injected with the same amount of any reasonable dose of the active material. Since the correlation between the increase of the dose of the injected hormone and the average degree of increase of prostate with seminal vesicles is one of direct proportion, the quotient of division of the average increase of the prostate and seminal vesicles by 40 will be the number of rat units injected (see Table V). In our experiments a test, from this point of view, was made only of doses from 1·0 to 7·5 RU. Whether the direct proportional correlation will remain true for much higher doses has yet to be investigated.

Summary.

1. Using 42 castrated rats belonging to 8 litters a test was made of the method of assay of testicular hormone and the suitability of the rat unit, as defined in previous papers, and a comparison was made betwen the rat unit and the capon unit.

2. From the results obtained, both the method used and the suggested rat unit seem to be satisfactory for the assay of testicular hormone.

3. The rat unit suggested is the minimum daily dose, which, when injected twice a day in half doses during 7 consecutive days, into at least 3 litters of rats castrated before the 30th day after birth, will produce on the average an increase of 40 % in the weight of the prostate with seminal vesicles as compared with the average weight of these organs in the uninjected litter-mates. For the assay it seems most convenient to use rats about 30 to 50 days after castration.

4. The rat unit proved to be nearly equal to the capon unit (as assayed in the Schering Laboratories).

5. On the average, a correlation of direct proportion was found between the increase of the dose, from 1 to 7·5 RU, and the degree of average increase in the weight of prostate with seminal vesicles and, less sensitively, of the penis.

6. For each RU or CU the increase in weight of the penis was about 18 % (actual weight) or 15 % (when calculated per 200 g. of body weight) as compared with that of the controls.

7. The response of the thymus to the injections of the hormone was variable and less sensitive.

8. Although, for assaying testicular hormone, a rat unit is suggested which is based on the more sensitive response of the prostate with seminal vesicles, it is also useful to note the changes in weight of the penis and thymus as supplementary proofs of the activity of the preparation.

9. The precautions which are necessary for obtaining accurate results in the assay are detailed, with special reference to the weight and age of the rats used for assay.

10. The disadvantages and advantages of the proposed method of assay and of the rat unit are discussed.

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CCL. THE REACTIONS OF LEAD COMPOUNDS WITH SERUM AND SERUM-MODELS[1].

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The reactions of lead compounds with serum are of interest, since they determine largely the fate of lead entering the body.

The first fairly direct evidence that lead ions react with the phosphate ions of serum was apparently obtained by Aub and his co-workers [1924], who found that haemolysis in hypotonic salt solutions was lessened by addition of soluble lead salts. Lead salts previously mixed with serum had no such effect. At the concentration present in serum, phosphates were the only substances found to have the same power of inhibiting the action of lead as had serum itself.

Scremin [1925] added solid lead iodide, carbonate and sulphate to serum, and found phosphate in the sedimented precipitates.

Brooks [1927] ultrafiltered serum and aqueous solutions after adding small quantities of lead acetate or of a suspension containing metallic lead and lead hydroxide. The lead in the mixtures was not filterable, and some of the phosphate had ceased to be filterable. The observed mean ratios of phosphorus atoms removed from solution to lead atoms added are given in Table I.

Table I. *Data of Brooks.*

	Filtered	P/Pb
Lead acetate added to phosphate + NaCl	At once	0·62
Lead suspension added to phosphate + NaCl	After 7 days	0·62
Lead acetate added to Ringer's solution	At once	0·69
Lead suspension added to Ringer's solution	After some days	1·0
Lead acetate added to cow serum	After some days	1·1
Lead suspension added to cow serum	After some days	1·1

These results were interpreted by Brooks as indicating formation of $PbHPO_4$ or $Pb_3(PO_4)_2$, which would require ratios of 1·00 or 0·67.

Bischoff and Maxwell [1928] made similar experiments, including however analyses for calcium, and found that calcium also became unfilterable. Figures calculated from their experiments are given in Table II. They show that calcium precipitates with the lead, except in acid solutions.

[1] This investigation was undertaken on behalf of the Liverpool Medical Research Organisation (Director: Prof. W. Blair Bell).

Table II. *Data of Bischoff and Maxwell.*

	Ca/Pb	P/Pb	P/Ca+Pb
Lead acetate added to Ringer's solution	0·28†	0·67	0·52
Lead hydroxide added to Ringer's solution	0·73	0·90	0·52
Lead acetate added to phosphate + $CaCl_2$*	0	0·70, 0·63	—
Lead acetate added to rabbit serum	1·03	1·21	0·60
Lead acetate added to rabbit serum	0·92	1·39	0·72
Lead acetate added to rabbit serum	0·92	1·19	0·59
Lead acetate added to pig serum	0·94	1·37	0·71
Mean of serum experiments	0·95	1·29	0·655

* The final p_H was <6·0. In the other experiments it was >7·0.
† The low value is due to a shortage of calcium in the solution.

In serum the data, as interpreted by the authors, would indicate that probably $Pb_3(PO_4)_2.Ca_3(PO_4)_2$ is formed, since this would require the ratios in the table to be 1·00, 1·33 and 0·67. Bischoff and Maxwell assume, however, that only filterable calcium reacts with phosphate, although they themselves show that non-filterable calcium can disappear by reaction with oxalate. If we assume instead that the partition of calcium between the forms initially present is unchanged by addition of lead, we can derive from their data the ratios 1·33, 1·29 and 0·56, which no longer approximate to the requirements of $Pb_3(PO_4)_2.Ca_3(PO_4)_2$.

The exact product of the addition of lead salts to serum is therefore still uncertain, and the present work was undertaken to obtain further information on its nature, and on the rates of the reactions, which are of toxicological interest. It seemed improbable that the ultrafiltration method already used would yield much more of interest, and the author has employed a manometric method, which is less specific but more suited to measuring rates of reaction. The lead electrode has also been of value.

A less direct method of attack follows from the general tendency for the formation in precipitations of the least soluble salt. Measurements of the solubility of lead carbonate will be given before other salts are considered.

The solubility of lead carbonate.

Measurements of the solubility of lead carbonate in aqueous solutions at 37·5° were made by the manometric methods of Warburg [1926]. As the results are not very accurate, they are reported briefly.

2–4 cc. of dilute solutions of lead acetate, containing sometimes a little added HCl or $NaHCO_3$, were shaken in manometric vessels in the presence of nitrogen containing about 5 % CO_2. Lead carbonate precipitates according to the equation

$$Pb^{++} + CO_2 + H_2O \rightarrow PbCO_3 + 2H^+.$$

After some hours excess of HCl is added from a separate compartment in the vessel; the lead carbonate redissolves, and the CO_2 previously absorbed is evolved. The increase in pressure tells us the amount of $PbCO_3$ that had precipitated—some 30–40 % of the total lead—and hence the amount of Pb^{++}

left in solution. We can also calculate the amount of H^+ that has been liberated; this reacts almost completely with acetate ion to give acetic acid. From the ratio of acetic acid to acetate in the system we obtain the p_H, which in turn regulates the concentration of $CO_3^=$. The solubility product of lead carbonate can therefore be calculated from one pressure change, provided that we know the initial composition of the solution, the CO_2 content of the gas mixture, and the various equilibrium constants and activity coefficients concerned. The presence of H^+ and HCO_3^- in the solutions has to be allowed for in the calculations, which are made by successive approximations.

Experiments were also made in the presence of a suspension of lead carbonate. After some hours' shaking $NaHCO_3$ is added instead of HCl, and the Pb^{++} left in solution is estimated by the gas change in the reaction:

$$Pb^{++} + 2HCO_3^- \rightarrow PbCO_3 + CO_2 + H_2O.$$

Similar values were obtained by this method, with which equilibrium between solid and solution should be attained more rapidly. This indicates that equilibrium is probably attained by both methods.

The calculated solubility products are given in Table III. The solubility product S is defined by the relation

$$-\log_{10} S = pS = p\mathrm{Pb}^{++} + p\mathrm{CO}_3^=,$$

in which the ionic terms are activity terms[1].

Table III. *Solubility product of $PbCO_3$ at 37·5°.*

Ionic strength	pS
0·020	11·91
0·019	12·00, 11·88, 12·00, 12·00
0·017	12·00
0·012	12·18, 12·23, (12·02)
0·009	12·24, 12·23, (11·64), 12·23, 12·24
0·008	12·22, 12·24
0·005	(12·42)
0·004	12·16, 12·22

From the values at the lower ionic strengths we take a mean value 12·22 for pS, which may be in error by 0·2, since equilibration of the solutions with accurately known gas mixtures was not very exactly attained. No other measurements appear to have been made at 37·5°.

[1] The equilibrium constants assumed are as follows:

$$\frac{a_{H^+} \times a_{CH_3COO^-}}{C_{CH_3COOH}} = 10^{-4.75},$$

$$\frac{a_{H^+} \times a_{HCO_3^-}}{C_{CO_2}} = 10^{-6.33} \text{ [Hastings and Sendroy, 1925].}$$

$$C_{CO_2} = \frac{0.56}{22.3} \times \frac{P}{760} \text{ [P = partial pressure of } CO_2 \text{ in mm. Hg],}$$

$$a_{CO_3^=} = 10^{-16.55} \frac{C_{CO_2}}{a_{H^+}^2} \text{ [Hastings and Sendroy, 1925].}$$

Activity coefficients for Pb^{++}, CH_3COO^- and HCO_3^- are calculated by the simple Debye-Hückel equation. The equilibrium lead ion concentration is about $\frac{1}{3}$ the ionic strength.

Solubilities of certain lead salts in serum.

We shall now calculate the solubilities in human serum at 37·5° of certain lead salts, the anions of which are present in serum. An approximate value for the solubility product of lead carbonate has just been obtained. For secondary and tertiary lead phosphates, and pyromorphite, we make use of solubility products derived by Jowett and Price [1932] for aqueous solutions at 37·5°.

We take the p_H of serum to be 7·35, its phosphate concentration to be 0·0012 M, and the bicarbonate 0·025 M. The values in Table IV for the solubilities of the lead salts in serum can then be derived[1], in terms of the lead-ion concentrations at equilibrium.

Table IV. *Solubilities of lead salts in serum.*

Salt	Lead-ion concentration	Solubility ratio	P/Pb
$PbCO_3$	$2{\cdot}6.10^{-7}$ M	1000	0
$PbHPO_4$	$1{\cdot}7.10^{-7}$ M	660	1·00
$Pb_3(PO_4)_2$	$1{\cdot}2.10^{-8}$ M	50	0·67
$Pb_5Cl(PO_4)_3$	$2{\cdot}6.10^{-10}$ M	1	0·60

The least soluble salt by far is pyromorphite, $Pb_5Cl(PO_4)_3$ or $PbCl_2 . 3Pb_3(PO_4)_2$. This is therefore, of these salts, the most likely to be formed when lead salts are added to serum or serum-like solutions, and will be the ultimate reaction product if these salts are the only possible resultants.

In pyromorphite the atomic ratio of phosphorus to lead is 0·60. Brooks found (Table I) a ratio of 0·62 for lead compounds added to a phosphate-chloride mixture, and hence the compound formed was probably pyromorphite, where Brooks believed it to be the tertiary phosphate of lead.

On adding lead chloride to a similar mixture containing also bicarbonate, the present author found a phosphorus-lead ratio of 0·56, by analysis after an hour or two. Probably some lead carbonate was precipitated, the ratio thus being lowered below 0·60.

When Ca^{++} is present, the precipitate contains calcium, and the atomic ratio of calcium to lead is of the order unity. Carbonate may be present in the precipitates, since with Ringer's solution an atomic ratio of 0·52 for P/(Ca + Pb) is found (Table II). The precipitates however probably consist mainly of phosphorus-containing compounds, and we have to decide between compounds of the type $(Pb, Ca)_3(PO_4)_2$ and of the type $(Pb, Ca)_5Cl(PO_4)_3$. The following arguments favour the latter type of formula.

(1) The reaction product in solutions containing no calcium is probably mainly pyromorphite. Pyromorphite is much less soluble than $Pb_3(PO_4)_2$, and

[1] p_{K_2} and p_{K_3} for phosphoric acid are assigned the values 7·165 and 12·18, following Bjerrum and Unmack [1929], and p'_{K_2} for phosphoric acid in serum is assumed to be 6·72 (see later). $\gamma_{HPO_4^=}$ is taken to be 0·235. Hence $a_{HPO_4^=} = 10^{-3{\cdot}64}$ and $a_{PO_4^{\equiv}} = 10^{-8{\cdot}47}$, and the calculated figures may be obtained, taking $\gamma_{Pb^{++}}$ to be 0·11, the value used by Jowett and Price [1932] in 0·16 M NaCl.

the entry of calcium into the structures would probably leave the pyromorphite type of solid still the less soluble.

(2) The P/(Ca + Pb) ratio recalculated by the author from the data of Bischoff and Maxwell in serum, 0·56, and the ratio found by these authors in Ringer's solution, 0·52, both favour a substance of ratio 0·60 rather than 0·67.

(3) Pyromorphite occurs in a number of minerals, lead being partly replaced by various metals, including calcium; $Pb_3(PO_4)_2$ is of very limited occurrence in nature. Lead-calcium compounds of the pyromorphite type are therefore known to exist, while those of the tertiary phosphate type are not.

(4) Chloro-apatite, $Ca_5Cl(PO_4)_3$, has also a wide distribution in nature while $Ca_3(PO_4)_2$ has not. On the other hand it seems likely that the calcium compound uniting with lead might be similar to one of those formed in ossification; chloro-apatite may be present in bones [Bassett, 1917] but the small chloride content of bones shows that it is not largely present.

The manometric method.

In most of the reactions which lead compounds are likely to undergo in serum or serum models acid-base changes occur. In systems buffered by CO_2 and HCO_3^-, these changes may be followed by measurements of the pressure of carbon dioxide.

Pressure changes have been followed by the type of manometers and vessels employed by Warburg [1926]. The vessel attached to the manometer has two compartments. Into the small compartment we measure 0·1–0·4 cc. of the lead-containing solution, into the larger one 1–4 cc. of serum or other liquid. The vessel is swept out with a gas-mixture containing some 5 % CO_2 in nitrogen, and is then shaken in a thermostat at 37·5° until the manometer level is steady. The contents of the two compartments are mixed by tilting the vessel to and fro, and pressure readings are then made at intervals, the vessels being shaken between readings.

The reactions of lead ion with serum-models.

A. In absence of calcium. When lead chloride is added to a neutral solution containing chloride, bicarbonate and phosphate, one of the following precipitations would be expected to occur:

(1) $Pb^{++} + HPO_4^{=} \rightarrow PbHPO_4$,
(2) $Pb^{++} + HCO_3^- \rightarrow PbCO_3 + H^+$,
(3) $3Pb^{++} + 2HPO_4^{=} \rightarrow Pb_3(PO_4)_2 + 2H^+$,
(4) $5Pb^{++} + 3HPO_4^{=} + Cl^- \rightarrow Pb_5Cl(PO_4)_2 + 3H^+$.

Owing to the buffering of the phosphate and carbonate-bicarbonate systems additional changes take place, but to a first approximation the H^+ released according to these equations would liberate an equivalent amount of carbon dioxide. Reaction (1) would liberate no gas, and the other reactions 1, 0·67 or 0·60 moles of CO_2 per mole of Pb^{++}.

The results obtained manometrically are indicated in Exps. 1–4 of Table V. It is assumed in the calculations that the evolution of carbon dioxide is due to combination of Pb^{++} with $PO_4^{\equiv}$ according to such equations as (3) or (4), allowance being made for the buffering effects. The ratio of P disappearing

Table V. *Addition of lead chloride to aqueous solutions.*

Exp. No.	Initial concns. (*mM*/l.) of Ca	Phosphate	$NaHCO_3$	p_H	Moles $PbCl_2$ $\times 10^6$	Vols. of solutions	$mm.^3$ CO_2	P/Pb
1	0	1·5	24·6	7·50	2·4	0·24, 4·0	36·9	0·68
2	0	1·5	26·1	7·31	3·0	0·2, 3·5	44·5	0·59
3	0	0·75	16·4	7·32	3·0	0·3, 4·0	48·0	0·62
4	0	1·5	24·6	7·50	4·0	0·4, 4·0	57·4	0·62
5	1·25	1·46	24·0	7·49	1·2	0·12, 4·0	35·6*	1·26*
6	1·25	1·46	24·0	7·49	1·2	0·12, 4·0	33·8	1·20
7	1·25	1·46	24·0	7·49	2·5	0·25, 4·0	55·5	0·95
8	2·5	1·42	23·4	7·48	2·5	0·25, 4·0	77·6	1·29
9	5·0	1·35	22·2	7·45	2·45	0·245, 4·0	116·0	1·90
10	1·12	1·47	25·7	7·30	3·0	0·20, 3·58	63·9	0·83
11	1·66	1·45	25·4	7·29	3·0	0·20, 3·62	67·2	0·86
12	3·21	1·40	24·6	7·28	3·0	0·20, 3·74	86·6	1·10

NaCl was also present, bringing the ionic strength to 160 *mM*/l.

* In Exps. 5–9 the values given are those obtained 5 hours after the addition, in Exps. 10–12 1 hour after.

to Pb added is found to be about 0·62, the same value as that of Brooks. Both values may be somewhat in error (the manometric value by perhaps 3–5 %) but they indicate that the product formed is probably mainly pyromorphite. The agreement of the two values rules out much formation of $PbCO_3$, the probable formation of which in experiments referred to earlier may be due to conditions less favourable to attainment of equilibrium.

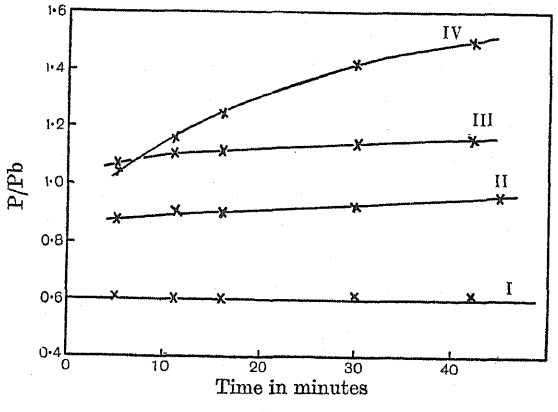


Fig. 1. Addition of lead chloride to Ringer's solutions.

The evolution of gas after addition of lead in terms of the P/Pb ratio is shown by Curve I, Fig. 1 (Exp. 4), and by Curve I, Fig. 2 (Exp. 2). It is complete in about 5 minutes, and takes place at about the same rate as when HCl is added to Ringer's solution. The precipitation must therefore be rapid—a

turbidity is visible immediately—the lag in evolution of gas being due to the limited surface and rate of shaking of the vessels.

B. In presence of calcium. When lead chloride is added to solutions of the Ringer type containing calcium, the reactions are more complicated. The results (Table V, Exps. 5–12) have been calculated as before.

The evolution of gas is rapid for about 5 minutes and continues at a diminishing rate for some hours. Curves II and III of Fig. 2 show the evolution

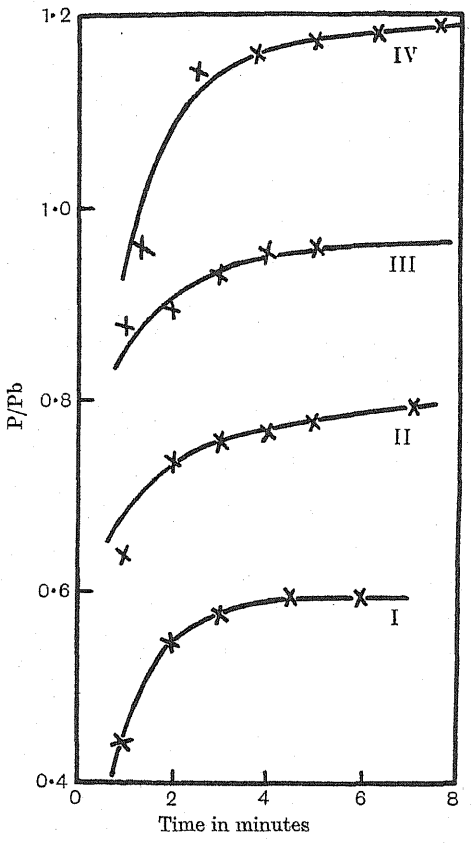


Fig. 2. Addition of lead chloride to Ringer's solution or serum.

of gas in Exps. 11 and 12, corrected for the lag of evolution on the assumption that the lag is the same as in similarly conducted experiments in absence of calcium, the chemical reaction being taken to be instantaneous in the latter case. The curves represent the extent to which Pb^{++} has reacted with $PO_4^{\equiv}$ at various times. After a very short time the P/Pb ratio exceeds 0·60, and is higher for the system in which the initial calcium ion concentration is greater. In Fig. 1 are shown gas evolutions, expressed in terms of P/Pb ratios, over greater time intervals: Curves II, III and IV illustrate Exps. 7, 8 and 9. The initial $[Ca^{++}]$ rises from Exp. 7 to 9, and so does the P/Pb ratio.

In Table VI the data are arranged to show that the initial Ca^{++}/Pb^{++} ratio in the solution, before precipitation has begun, determines largely the value of P/Pb that is reached after some time.

Table VI.

Exp. No.	P/Pb	Initial Ca^{++}/Pb^{++}	Initial Ca^{++}	$0{\cdot}60+0{\cdot}155$ Ca^{++}/Pb^{++}
10	0·83	1·3	1·12	0·80
11	0·86	2	1·66	0·91
7	0·95	2	1·25	0·91
12	1·10	4	3·21	1·22
6	1·20	4·2	1·25	1·25
5	1·26	4·2	1·25	1·25
8	1·29	4	2·50	1·22
9	1·90	8·2	5·00	1·88

The findings may be interpreted as follows. As in the absence of calcium, pyromorphite, $Pb_5Cl(PO_4)_3$, precipitates rapidly. Calcium is however able to compete with lead for a place in the molecular structure, and the actual substance produced is $(Pb, Ca)_5Cl(PO_4)_3$. The extent to which calcium can enter the precipitate by competition with lead depends on the Ca/Pb ratio in the solution. This ratio rises as precipitation proceeds, but the initial value serves as a measure of the effect. The ratio of phosphorus disappeared to lead added therefore reaches a value 0·60 or higher very rapidly: the extent to which the value is higher than 0·60 depends on the Ca^{++}/Pb^{++} ratio, and is roughly proportional to it, as shown in the last column of Table VI. After the initial rapid precipitation of calcium and lead, the removal of phosphate from the solution continues, but at a much slower rate. Aggregation of the precipitate may make it more difficult for Ca^{++} to react with it: insolubility of the precipitate may tend to prevent replacement of Pb by Ca, and there must be a limit to the extent to which Ca can add on to the precipitate to form a layer containing little or no Pb.

The above view of the phenomena is not completely satisfactory. It does not explain why the reaction continues so much more rapidly in Exp. 9 (Curve IV, Fig. 1) than in the others.

An alternative explanation of the facts can be proposed. Calcium tends to add on to the precipitated $Pb_5Cl(PO_4)_3$ until it is converted into $Pb_5Cl(PO_4)_3.Ca_5Cl(PO_4)_3$. In support of this, the P/Pb ratio reaches values near 1·20 in four experiments.

The single very high value for P/Pb can be explained by supposing that in Exp. 9, where the $[Ca^{++}]$ is the highest of the series, precipitation of $Ca_3(PO_4)_2$ takes place. All the solutions are supersaturated with respect to $Ca_3(PO_4)_2$, that in Exp. 9 being most highly supersaturated. In Exp. 9 some 90 % of the phosphate is removed from solution, and hence shortage of phosphate may be the limiting factor to reaction.

We have hardly sufficient data to decide between the two possibilities raised: (1) that the Ca/Pb ratio in the precipitate can vary continuously, and

(2) that the Ca/Pb ratio tends to rise to unity and stop there, unless calcium phosphate should precipitate as well.

The second explanation appears rather more probable, taking into account also the results obtained with serum.

Calculation of results.

The details of the calculation for Exp. 4 are given below. The terminology of Warburg [1924; 1925] is employed. Pressures are expressed in such a unit that 10,000 mm. equal 1 atmosphere, and concentrations often in mm.^{3} per cc., where 22,300,000 mm.^{3} equal 1 mole[1].

An hour after adding 0·4 cc. of 0·01 M $PbCl_2$ to 4·0 cc. of solution, the increase in pressure was 50·2 mm., corresponding to an evolution of 57·4 mm.^{3} of CO_2 in the system:

$$\Delta h = \Delta p_{CO_2} = +\ 50{\cdot}2. \quad k^{\text{Ring.}}_{CO_2} = 1{\cdot}144,\ x_{CO_2} = 57{\cdot}4\ (\text{mm.}^3).$$

The initial pressure of CO_2 was 420 mm. (approximately, from the also approximate composition of the gas-mixture), and the final pressure is therefore 470·2: $p_{CO_2} = 420$, $p'_{CO_2} = 470{\cdot}2$.

The initial $[HCO_3^-]$ (0·0246 M) is given by:

$$x_{HCO_3^-} = 0{\cdot}0246 \times 1{\cdot}10^{-3} \times 2{\cdot}23.10^7 = 550\ (\text{mm.}^3/\text{cc.}).$$

When both dilution and evolution of CO_2 are taken into account, the final $[HCO_3^-]$ concentration is 487:

$$x'_{HCO_3^-} = \frac{550 \times 4 - 57{\cdot}4}{4{\cdot}4}.$$

The p_H values are now calculated by means of the equation[2]

$$p_H = 6{\cdot}13 - \log \frac{0{\cdot}056\, p_{CO_2}}{x_{HCO_3^-}},$$

which yields the values 7·499 and 7·397. The p_H thus decreases during the reaction by about 0·102.

The ratio of $HPO_4^=$ to $H_2PO_4^-$ in the solution is altered by the change of p_H. The amounts of the ions are decreased by the precipitation, and to calculate a trial result we assume that the moles of phosphate disappearing are 0·60 times the g.-atoms of lead added. The phosphate initially in solution in the vessel is $6{\cdot}10^{-6}$ moles, and finally (on our assumption) $3{\cdot}6.10^{-6}$.

We calculate the moles of $H_2PO_4^-$ and $HPO_4^=$ initially and finally present by means of the relation[3]

$$p_H = 6{\cdot}72 + \log \frac{C_{HPO_4^=}}{C_{H_2PO_4^-}},$$

[1] Warburg uses the value 22,400,000, but according to E. J. Warburg [1921] the lower value is more accurate for CO_2.

[2] The value 6·13 is that used by Hastings, Murray and Sendroy [1926–7].

[3] The data of Bjerrum and Unmack [1929] yield the value 6·726 for solutions of ionic strength 0·16, which however do not consist mainly of sodium chloride. Using the p_{K_2} value of these authors, namely 7·165, and taking a rather inaccurate value 0·458 for $\Delta p_{K'_2}$ for isotonic salt solutions from the data of Jowett and Millet [1929], we derive a value 6·707. Sendroy and Hastings [1926–7] use the value 6·66 for serum, but the value 6·72 is preferred here for serum also.

and obtain the values $8{\cdot}56.10^{-7}$ and $6{\cdot}26.10^{-7}$ for $H_2PO_4^-$, and $51{\cdot}44.10^{-7}$ and $29{\cdot}74.10^{-7}$ moles for $HPO_4^=$. Hence $2{\cdot}3.10^{-7}$ moles of $H_2PO_4^-$ disappear and $21{\cdot}7.10^{-7}$ of $HPO_4^=$. Each mole of $H_2PO_4^-$ reacting with lead releases 2 moles of CO_2, and each mole of $HPO_4^=$ 1 mole of CO_2.

The CO_2 appearing in the system should therefore be given by

$$= 2{\cdot}23.10^{-7} \times (21{\cdot}7.10^{-7} + 2 \times 2{\cdot}3.10^{-7}) = 58{\cdot}7.$$

A correction must be applied to this value, since a small amount of $CO_3^=$ is present in the solution, and by the dilution and acidification some of this is transformed to HCO_3^- with absorption of CO_2.

The concentration of $CO_3^=$ is given by[1]

$$x_{CO_3^=} = \frac{(x_{HCO_3^-})^2}{227\, p_{CO_2}}.$$

Hence $x_{CO_3^=}$ is initially 3·17, and finally 2·22; the amount of $CO_3^=$ (and CO_2) disappearing from the system is therefore 2·9 mm.[3]

Hence the calculated formation of CO_2 in the system is 55·8, while the observed value is 57·4. The observed value for P/Pb is therefore not 0·60, but 0·62.

The other experiments have been calculated similarly; the trial value for P/Pb must be near the observed.

The reaction of lead ion with serum.

When lead chloride is added to cow-serum, the results are very similar to those which follow its addition to Ringer's solution containing calcium. The evolution of gas becomes slow after 5 minutes[2], but continues for perhaps an hour. The rate of the slow process is of the same order as in Ringer's solution, although the particles formed precipitate in Ringer's solution after a few minutes, and do not in serum. The favouring influence of small particle size in serum may be offset by protein adsorption hindering reaction.

The buffering of serum results in formation of CO_2 from only about a half of the H^+ developed in the system. The calculation of the acid formed (x_s) has been made by the methods of Warburg [1925], using the constants for carbonic acid of Hastings, Sendroy and Van Slyke [1928]. The ratio of $H_2PO_4^-$ to $HPO_4^=$ reacting with lead is determined by the final p_H of the system. As before, the data have been interpreted on the assumption that Pb^{++} reacts with $PO_4^{\equiv}$, and the ratios of P/Pb derived from the gas evolved after 60–90 minutes are shown in Table VII. Three different samples of serum were used, the last one being kept in a refrigerator for 3 days before use.

The observed values of P/Pb may be in error by some 10 %, and the variations shown in Table VII may not be real. The mean observed value is

[1] The value used for the equilibrium constant has been interpolated from the data of Walker, Bray and Johnston [1927]; it is 102 in these authors' units, 227 in ours.

[2] Shown in Curve IV, Fig. 2, which illustrates Exp. 3*a*; the rate of evolution is corrected to that due to chemical reaction by taking lag due to physical causes to be the same as when HC is added to serum.

1·19. By direct analysis Brooks found a value 1·1, and Bischoff and Maxwell 1·29. As the manometric value lies between these, we have evidence that the main process actually occurring is disappearance of $PO_4^{\equiv}$, and the value is in agreement with the view that the product of reaction is the compound $Pb_5Cl(PO_4)_3 . Ca_5Cl(PO_4)_3$.

Table VII. *Addition of lead chloride to cow-serum.*

Exp. No.	Moles $PbCl_2$ $\times 10^6$	Vol. of solutions	x_s	Final p_H	P/Pb
1*a*	2·5	0·25, 3·0	74·6	7·04	1·01
1*b*	2·5	0·25, 3·0	79·3	7·04	1·07
2*a*	2·93	0·195, 3·5	94·4	7·17	1·15
2*b*	3·0	0·20, 3·5	102·2	7·17	1·21
3*a*	3·0	0·20, 3·5	115·0	7·19	1·37
3*b*	3·0	0·20, 3·5	110·9	7·19	1·32

The calcium contents of the sera were not estimated. The phosphate concentration in one case was 0·0023*M*. Both calcium and phosphate concentrations were probably similar to those in the aqueous solutions in which P/Pb reached values near 1·2, and our experiments do not show evidence of any differences between the reaction in Ringer's solution and serum, except that in serum there are no values which can be attributed to formation of calcium phosphate. This agrees with the fact that injections of lead preparations do not cause cardiac symptoms similar to those produced by injections of colloidal calcium phosphate.

The reactions of other lead compounds.

Experiments were made with the colloidal preparation S_7, described by Brooks [1927], and with similar preparations containing no calcium. The colloids, which contain mainly metallic lead and lead hydroxide, were treated with oxygen to oxidise the lead, centrifuged slightly, and added (as before) to phosphate-containing solutions in absence or presence of calcium chloride, or to serum.

Some carbon dioxide from the gas mixture was however absorbed by the alkaline colloids before they were mixed with the solutions (although the colloid was covered with liquid paraffin) and as the absorption was variable the manometric data can only be interpreted semi-quantitatively. As far as they go, they show that the products of reaction are the same as when lead chloride reacts.

In the absence of calcium the manometric change is about 90 % complete in 5 minutes: the proportion of lead that has reacted will be less than this, since lead carbonate in reacting brings about less gas exchange than does lead hydroxide, which is likely to react more rapidly. The reaction when calcium is present is also rapid. The rapidity of the reactions must be due to the small size of the particles. It is intelligible that calcium should react well with the particles: the $[Pb^{++}]$ at the reacting surface has as an upper limit the

solubility of lead hydroxide (about 0·0005 M), while the [Ca^{++}] in the solution is 0·0025 M.

Lead carbonate when finely divided—prepared by adding oxidised S_7 to a solution containing bicarbonate—also reacts rapidly with phosphate, the reaction being about half complete in 5 minutes. Brooks found the reaction to be slow when a coarse suspension was used.

The slow completion of the reaction of S_7 with phosphate, which Brooks attributed to slow reaction of lead carbonate with phosphate, is probably due to a slow completion of the reaction of calcium.

Experiments have been made with two further preparations of lead, (1) a colloidal solution of the lead salt of benzenesulphonylglycine[1], and (2) a soluble compound[2] known as R 232, in solutions of which a very low concentration of Pb^{++} is maintained in equilibrium with a lead compound. These compounds react with phosphate in absence and presence of calcium in a very similar manner to lead chloride, the rates of reaction being similar.

Summing up, we may say that Pb^{++} reacts almost instantaneously with phosphate, and so do fairly soluble lead salts (lead hydroxide and lead benzenesulphonylaminoacetate). The less soluble lead carbonate reacts rapidly if finely dispersed, and the very insoluble lead sulphide reacts very slowly [Brooks, 1928]. The reactivity of lead compounds evidently depends on the rate at which they can form Pb^{++}, solubility and surface both being of importance. No other factors have yet manifested themselves.

Lead ion concentrations during the reactions.

Lead ion concentrations have been measured after addition of lead compounds to solutions of the Ringer type. Measurements were made by the technique of Millet [1929], standardisation in terms of [Pb^{++}] being effected by use of a standard solution[3].

1 cc. or less of S_7 or dilute lead chloride is added to some 25 cc. of solution (containing 0·025 M $NaHCO_3$, and of p_H 7·55–7·6), and after the solutions are mixed E.M.F. measurements are begun at once. The solutions mixed are indicated in Table VIII, and the Pb^{++} concentrations during the first half-hour are shown in Fig 3.

Precipitation of Pb^{++} progresses so rapidly that before measurements are begun the Pb^{++} concentrations have fallen in every case below $1 \cdot 10^{-7}$ M: they continue to fall, but at a decreasing rate.

[1] This preparation, which contained gum acacia and sodium thiosulphate, was made by Prof. I. M. Heilbron and Dr B. Beilensohn in the Department of Organic Chemistry, University of Liverpool.

[2] Supplied to Prof. W. Blair Bell by Dr W. Collier, of the Koch Institute, Berlin.

[3] We assume here for simplicity that all the lead is present as lead ion. Probably association of Pb^{++} to $PbCl^+$ takes place, but its extent will be fairly constant in the solutions concerned. The lead amalgam electrode apparently functions correctly at these low Pb^{++} concentrations, owing to its regulation by a dispersed lead salt, and in the case of S_7 to removal of oxygen by metallic lead.

Table VIII. *Addition of lead compounds to various solutions.*

Curve	Compound added	*mM* Phosphate	*mM* Ca	Lowest lead ion concentrations observed: $pC_{Pb^{++}}$	Time (mins.)	Compound being formed
VI	S_7	0	0	7·32	27	$PbCO_3$
V	$PbCl_2$	1	1	8·59	72	
	$PbCl_2$	1	1	9·00	280	$Pb_5Cl(PO_4)_3$
IV	S_7	1·5	1	8·96	42	$Ca_5Cl(PO_4)_3$
III	S_7	1·5	1	9·46	170	
II	S_7	1·5	0	9·54	66	
I	S_7	1·5	0	9·57	30	$Pb_5Cl(PO_4)_3$

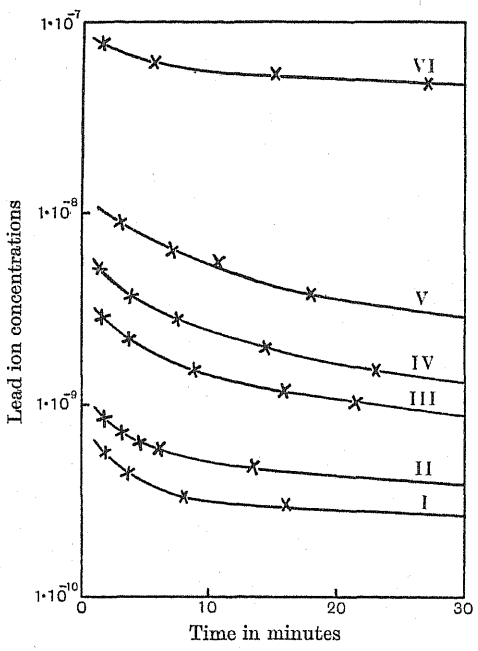


Fig. 3. Lead ion concentrations during the precipitations.

The experimental curves are widely spread out in Fig. 3, indicating that different reactions are occurring, since the $[Pb^{++}]$ must tend towards the solubility of the compound which has been formed.

The experiments were made at 25°, and our solubility data relate to 37·5°. The calculated equilibrium Pb^{++} concentrations (at 37·5°) for our experimental conditions are for $PbCO_3$ $10^{-6·8}$, for $Pb_3(PO_4)_2$ $10^{-8·1}$, and for $Pb_5Cl(PO_4)_3$ $10^{-9·8}$. These figures are fortunately widely different.

When no phosphate is present we should expect formation of $PbCO_3$, and this apparently takes place, for Curve VI lies at a Pb^{++} level of about 10^{-7}.

In no case is $Pb_3(PO_4)_2$ the final product of reaction, unless the temperature coefficient of the solubility of this salt is extremely high. Curves I–V are tending towards values below $10^{-9·5}$, and the reaction product is therefore

either $Pb_5Cl(PO_4)_3$ or a compound of similar solubility. This deduction is made from E.M.F. measurements alone, and is an independent confirmation of the conclusion already reached.

The slope of the curves is greater when calcium is present (III–V) than when it is absent (I, II). It might therefore be inferred from this alone that calcium takes some part in the reaction. With our previous knowledge, we may note in the curves a confirmation of the view already advanced that calcium and lead ions compete for places in the solid phase. So far we have regarded the effects on the calcium; now we see that the lead ion is affected, in that its concentration does not reach a low level as rapidly in the presence of calcium as in its absence.

The data do not tell us whether the lead-calcium compound is more or less soluble in our solutions than pyromorphite, since measurements were not pursued to attainment of equilibrium. On theoretical grounds, if the lead calcium compound is the stable one in presence of calcium, it must also be the less soluble in terms of lead ion, but need not be much less soluble.

Summary.

The reactions of lead compounds, and in particular Pb^{++}, with serum and serum-models have been studied by a manometric method, with the lead electrode and with the help of solubility data. The manometric method is not specific, but is valuable for measuring rates of reaction.

When $CO_3^{=}$ is the only precipitant ion, lead carbonate is formed. In the presence of phosphate (and chloride) the product is pyromorphite, $Pb_5Cl(PO_4)_3$. When calcium also is present, as in serum, the product approximates to $Pb_5Cl(PO_4)_3.Ca_5Cl(PO_4)_3$, an equimolecular compound of pyromorphite and chloro-apatite. Lead ion reacts very rapidly, but the union of calcium is less rapid than that of lead and may be incomplete under unfavourable conditions. Solubility and extent of surface determine the rate of reaction of solid lead compounds with phosphate.

An approximate value is obtained for the solubility product of lead carbonate at 37·5°. The solubilities in serum of $PbCO_3$, $PbHPO_4$, $Pb_3(PO_4)_2$ and $Pb_5Cl(PO_4)_3$ are calculated, pyromorphite being the least soluble of the four salts.

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CCLI. SOME EXTRACTS OF PARATHYROID GLANDS CONTAINING AN ANTI-GROWTH FACTOR. I.

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(*Received November 4th, 1932.*)

Previous work on the parathyroid gland [Collip, 1925; Collip and Clark, 1925; Hjort, Robison and Tendick, 1925] has shown the presence of a serum-calcium raising factor. From experiments on animals injected with a preparation of this gland one of us [Thompson, 1931] was led to suspect the presence of an anti-growth factor. It seemed possible that this activity might be due to the serum-calcium raising factor itself or to the presence of a specific anti-growth factor as an impurity in the extract. A series of extracts was therefore prepared with the intention of eliminating the Collip factor as far as possible. Collip and Clark [1925] have shown that the latter is precipitated at p_H 4·8. All extracts, however prepared, were adjusted to this p_H and allowed to stand until no further precipitation took place.

The preliminary extracts were prepared by the following methods.

(1) Aqueous extraction.

(2) Extraction with 95 % alcohol.

(3) Extraction with 80 % alcohol containing 5 % by weight of sulphuric acid, afterwards neutralising with sodium hydroxide to p_H 4·8, filtering off the sodium sulphate and concentrating *in vacuo*.

(4) Collip's method, heating with dilute hydrochloric acid, making alkaline to p_H 8 to 9, precipitating inert matter by bringing to p_H 5·6, acidifying to p_H 4·8 and allowing to precipitate.

(5) Method (4), but previously defatting the glands with acetone.

Acid alcohol extraction.

The results of animal tests with these fractions encouraged us to adopt method (3) as a basis for further investigations. Berman [1926] used a similar method for preparing an extract containing the Collip factor, but the acid alcohol extract was neutralised to litmus, thus retaining the Collip factor in solution. Incidentally it appears that Berman's extracts must have contained sodium sulphate, the alcohol concentration not being sufficiently high to remove it.

Method (3) was therefore elaborated for use with larger quantities of material. 100 g. of fresh parathyroid glands, freed from adherent fat and connective tissue and finely minced, were shaken continuously for 48 hours with 1 litre of alcohol (85 % by volume) containing 5 % by volume of sulphuric acid. The mixture of acid and alcohol must be made slowly and the temperature kept low, otherwise sodium ethyl sulphate is formed which appears in the final product. This compound has been found to have no growth-retarding action on rats.

The gland residue was then strained off, and the liquid was neutralised to methyl red by the gradual addition of 50 % sodium hydroxide solution, keeping the mixture cool. Alcohol was then added so as to give a final concentration of 75 % by volume in the mixture. This ensures the almost complete precipitation of the sodium sulphate, on cooling to about 5°. After keeping at this temperature overnight the sodium sulphate and other precipitated material were removed by filtration through a Büchner funnel. The filtrate was concentrated under reduced pressure at about 15–20 mm. until all the alcohol was removed. The volume of the concentrated liquid was adjusted so that 1 cc. was equivalent to 2 g. of parathyroid gland, and the p_H was adjusted

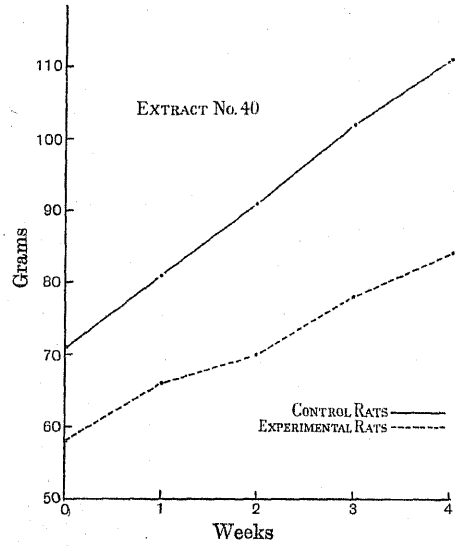


Fig. 1.

to 4·8. The extract was then allowed to stand at a temperature of about 5° until no further precipitation occurred, when it was filtered through a Seitz filter. The extract was used in this condition for animal tests without the addition of a preservative. For a description of the method of testing and for the physiological evidence for the anti-growth factor see Robinson and Thompson [1932].

A number of extracts prepared by this method have given definite evidence of the presence of an anti-growth factor. Fig. 1 shows the effect of such an extract on the growth of rats. Six rats were used as controls and six were given daily injections of 2 cc. of the extract, other conditions being the same.

Defatting by acetone. The large amount of fat in parathyroid glands prevents the efficient penetration of the gland material by the cold acid alcohol. Previous treatment of the minced glands with cold acetone appeared to give a more complete extraction. The fat so obtained appeared to possess a slight activity, as the following experiment shows.

200 g. of the fat extracted by acetone were dissolved in 750 cc. of light petroleum and shaken for 24 hours with 1 litre of 70 % by volume alcohol containing 10 % by volume of sulphuric acid. After separation the light petroleum layer was washed with 70 % alcohol and the combined acid alcohol extracts were treated as described above. The final concentration of the solution was, however, adjusted so that 1 cc. was equivalent to approximately 25 g. of gland instead of 2 g. The effect of this extract on rat-growth is shown in Fig. 2. The difference in the average increases in weight over 4 weeks is insignificant. Nevertheless if the last two weeks only be considered the growth of the injected rats was practically stationary during this period while the average increase of the controls was 13 g.

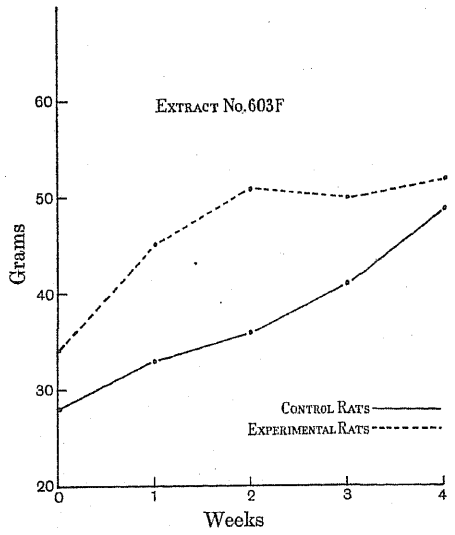


Fig. 2.

Defatting with benzene. In order to avoid this loss of active material benzene was added to the minced gland with the acid alcohol and the mixture was shaken together. This was an improvement on previous acetone extraction in removing the fat and allowing better extraction of the gland material.

400 g. of parathyroid glands were minced and shaken for 48 hours with

700 cc. of benzene and a cooled mixture of 300 cc. of sulphuric acid, 1800 cc. of water and 900 cc. of 95 % alcohol. The acid alcohol was separated and treated as in the first method.

Removal of fat by heat. The minced glands were mixed with alcohol (30 % by vol.) containing 5 % by vol. of sulphuric acid and heated to 70° for a few minutes until the fat was completely melted and rose to the surface. After cooling, the cake of fat was removed and the acid alcohol and gland material were shaken together for 48 hours. The liquid was filtered, neutralised with sodium hydroxide solution, and alcohol added to 75 % by vol. concentration as in the first method. The extract obtained was found to be active (see Fig. 3, extract No. 601). The fat obtained in this method was dissolved in benzene and extracted with acid alcohol. The results confirmed the previous finding that the fat contains some of the anti-growth factor.

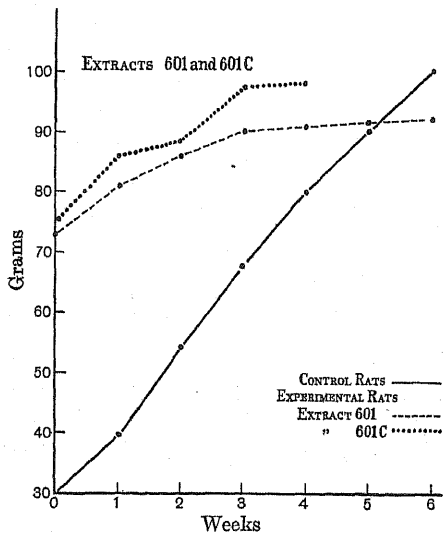


Fig. 3.

Salting-out. Extracts prepared by the above methods have been salted out with sodium sulphate at p_H 4·8 with a view to removing the Collip factor more completely. This has been repeated a number of times and in all cases in which it has been done an increase in the anti-growth potency has been observed. The usual procedure was to saturate the solution at p_H 4·8 with sodium sulphate at room temperature and filter off the precipitate formed, which was usually small in amount. The precipitate was redissolved in a smaller volume of water at p_H 4·8 and again salted out in the same way. The combined filtrates were treated with alcohol to a concentration of 75 % by volume and the sodium sulphate was filtered off after crystallising at 5° overnight. The alcohol was removed from the filtrate under reduced pressure and the concentrate was made up to the original volume with water.

The results on growth of such an extract (40 SO) are shown in Fig. 4.

The total average increase in the injected rats is only half the increase obtained with the extract before salting-out (40). Table I. Tests on a solution of the salted-out precipitate have failed to show any activity.

Alkaline extraction. 1200 g. of minced parathyroid gland were heated to 40–45° with 2400 cc. of 2 % sodium hydroxide solution and kept at that temperature for a few minutes, then at 37° for 24 hours, toluene being added as a preservative. After separation of the fat the liquid was neutralised to p_H 7 and centrifuged. The clear liquid was treated with alcohol until the concentration was 75 % by volume, the sodium sulphate was removed in the usual way, and the filtrate concentrated as usual. This extract appeared to have little or no activity.

Treatment with activated charcoal. 300 cc. of an active extract (601) were shaken with 6 g. of activated charcoal (Darco) for half an hour and filtered. The filtrate (601 C) still contained the anti-growth factor in undiminished amount (Fig. 3).

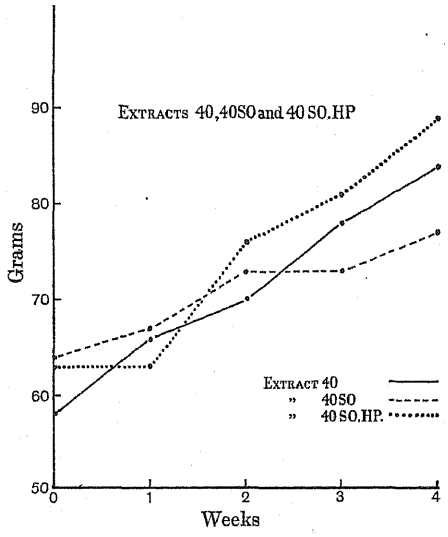


Fig. 4.

Treatment with hydrogen peroxide. 1 cc. of 30 % hydrogen peroxide was added to 300 cc. of an active extract (40 SO) and the mixture kept overnight at 22°. The excess of hydrogen peroxide was removed by the addition of manganese dioxide which was filtered off when the hydrogen peroxide was completely decomposed. This treatment appears in some cases to destroy the whole of the anti-growth activity [Robinson and Thompson, 1932] but the destruction is not always complete; for example, treatment with hydrogen peroxide reduced the potency of extract (40 SO) and the extract so obtained (40 SO.HP) appeared to have about the same potency as the original extract (40) before the salting-out process had increased its activity (see Fig. 4).

Chemical properties of the extracts. Extracts prepared by the acid alcohol method contain about 3 % of total solids, of which about 1·5 % are ash. The nitrogen content is about 0·2 %. Picric acid gives a slight precipitate, and phosphotungstic acid a heavy one. They give marked xanthoproteic and Millon's reactions which are reduced in intensity by the salting-out treatment and by treatment with activated charcoal. Ehrlich's reaction for tryptophan is also positive, but is very faint when applied to the extract after treatment with hydrogen peroxide. The extract of the fat was very low in solids and gave none of these reactions.

Summary.

1. Extracts have been prepared from parathyroid glands containing a factor which retards the normal growth of rats.

2. Extraction with acid alcohol gives active extracts.

3. Alkaline extraction gives little or no activity.

4. Removal of the fat gives better extraction, but the fat may contain some of the activity.

5. Salting-out increases the potency of active extracts, the salted-out material having no activity.

6. Treatment with activated charcoal does not affect the potency.

7. Treatment with hydrogen peroxide may partially or completely destroy the activity.

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CCLII. THE COLORIMETRIC DETERMINATION OF ADRENALINE IN SUPRARENAL GLAND EXTRACTS.

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Soon after the commencement of the manufacture of extract of suprarenal cortex for the treatment of Addison's disease it became evident that even moderate traces of adrenaline could not be tolerated owing to the large doses sometimes employed, and it became necessary to find a colorimetric method for the rapid estimation of adrenaline at certain stages of the process.

Various tests were tried on these comparatively crude extracts and the ammonium molybdate test, described later, was adopted provisionally since it was found that extracts which showed less than 1 part in 10,000 of adrenaline by this test, when injected, did not give rise to any of the physiological reactions characteristic of adrenaline.

With improved methods of manufacture much purer extracts were obtained which proved to contain no adrenaline when estimated biologically. Nevertheless, such extracts when tested colorimetrically with ammonium molybdate always gave some colour, usually equivalent to about 0·5 part per 10,000 of adrenaline but occasionally as high as 2 parts per 10,000. It was obvious, therefore, that it was useless to depend upon this test and it was decided to investigate further the subject of colorimetric determination of adrenaline in order to find a test which could be relied upon when applied to such gland extracts.

The numerous colour tests for adrenaline described in the literature have been grouped under three headings for the purposes of this investigation:

(*a*) those depending primarily on the formation of a red oxidation product;

(*b*) those depending on the presence of the catechol grouping;

(*c*) miscellaneous.

Included under heading (*a*) are the following:

(1) ferric chloride reaction, (2) potassium iodate reaction and its modifications, (3) potassium persulphate reaction, (4) mercuric chloride reactions, (5) other oxidising agents, including hydrogen peroxide, potassium ferricyanide, permanganate and dichromate, sodium bismuthate, sodium hypobromite, manganese dioxide and the halogens.

Under (*b*) we have included the ammonium molybdate test and Folin's test.

Miscellaneous tests include diazobenzenesulphonic acid, Azzolini's test [1931] and the ninhydrin test [Orru, 1929]. Another reaction (depending upon the formation of a blue ferrous-adrenaline complex) consists in the addition of a trace of ferrous sulphate followed by sufficient sodium phosphate (Na_2HPO_4, $12H_2O$) to bring the mixture to neutrality. A blue colour appears which is fairly stable but which rapidly fades through a purple colour if too much alkali is added. The reaction did not seem sensitive enough for the estimation of traces of adrenaline.

A preliminary examination of any test which appeared suitable for quantitative work was made and it was soon found that some of the tests described were extremely unreliable even when considered as qualitative tests, *e.g.* those described by Azzolini [1931], Paget [1931] and Orru [1929], this last being non-specific.

The reaction with mercuric chloride, originally suggested by Comessatti [1909] and modified by Ewins [1910], was not found to be very sensitive, although its application to gland extracts has been reported by Paget and Lohéac [1928] and Bailly [1924]. The modification of Comessatti's reaction described by Stuber, Russmann and Proebsting [1923] consists in the addition of a sulphanilic acid, potassium iodate and mercuric chloride reagent to adrenaline solution and boiling for 1 minute. They claim an extraordinary sensitivity of 1 in 100 millions for this test. Viale [1930] has applied this test to blood and confirms some of Russmann's statements. This test was, however, found to be unreliable, since the reagents themselves when mixed, on boiling produced an orange-brown colour which rapidly deepened on standing, due to the liberation of iodine. Purification of the sulphanilic acid produced no improvement and attempts to remove the excess of iodine with sodium thiosulphate were unsuccessful owing to the liberation of sulphur.

The reaction between adrenaline and diazotised sulphanilic acid described by Friend [1923] for quantitative application to urine (using a colorimeter) was also found unsatisfactory. No definite red colour was obtained either with pure adrenaline or with urine to which adrenaline had been added. Moreover, the orange-yellow colour which was obtained, while certainly somewhat stronger in the presence of adrenaline, was also given by a blank determination, making the colorimetric estimation of traces of adrenaline unreliable, if not impossible. The sulphanilic acid was recrystallised but still gave a yellow colour when ammonia was added to the diazotisation mixture. Further, the addition of ferric chloride to destroy the adrenaline present (as directed by Friend) did not alter the colour ratios obtained. This test is also described by Sansoni [1930] who states that it is the most sensitive and the easiest to employ, and although we were at a loss to account for its failure further attempts to utilise it were abandoned.

The remaining tests which were satisfactory qualitatively and appeared worthy of further investigation were Folin's test, the ammonium molybdate

test, and three oxidation reactions using ferric chloride, potassium iodate and potassium persulphate respectively as oxidising agents. Two of the latter were subsequently eliminated and will be dealt with first.

The ferric chloride reaction.

This reaction is characterised by the formation in slightly acid or neutral solution of a transient grass-green coloration, which changes to red on standing or by careful addition of dilute alkali. It is said that the more acid the solution the more fugitive and less marked is the green colour produced.

It was found that with pure adrenaline the green colour took about 10 minutes to change to red but that the reaction took place rapidly on boiling. A trial test was made using 2 cc. of a 1:100,000 adrenaline solution and 1 cc. of a 0·05 % $FeCl_3$ solution. The green colour produced on mixing was hardly discernible, but on boiling the solution and cooling, a colour of 1·8 red, 3·3 yellow Lovibond tintometer units was observed in a 1 cm. cell. The measurement of this orange-red colour seemed promising at the time but it was afterwards found that dilute $FeCl_3$ solution alone gives a somewhat similar colour on boiling, making the quantitative application of the test impossible. Further, it was found that suprarenal cortex extracts which contained no adrenaline gave definite reactions with this test.

It may be noted that alcohol does not interfere with this test and that if the colour obtained by addition of alkali be examined under ultra-violet light, using a Wood's screen, a very marked yellow fluorescence is observable very similar to that subsequently to be described under the ammonium molybdate test. The colour produced by adrenaline-free suprarenal cortex extracts does not show this property. The test is a very sensitive and reliable qualitative one for adrenaline but does not lend itself to quantitative application.

The potassium iodate reaction.

This test was originally applied quantitatively by Hale and Seidell [1911] who state that the method gives results 30 % below the biological value. Scoville [1920] who investigated the test much more fully found that the orange-red colour produced in neutral solution becomes reddish-violet in the presence of acid (acetic or hydrochloric) and prefers to do the test in acid solution (0·015 % hydrochloric acid) using an optimum temperature of 38° for 15 minutes. The method compares satisfactorily with the biological method provided the acidity of the original solution is known and reducing substances such as bisulphites (sometimes added as preservatives) are absent. By this method it is possible to estimate concentrations of adrenaline greater than 1 in 100,000 and the test is of about the same order of sensitivity as the persulphate test. Moreover, suprarenal cortex extracts free from adrenaline give no colour and the reaction should prove capable of measuring adrenaline in such extracts with a high degree of accuracy.

As, however, the reaction is essentially very similar to the persulphate reaction which is described later and as the latter is easier to use, the test was not further investigated.

The ammonium molybdate reaction.

This test, described by Rae [1930], depends on the presence of a catechol grouping in the adrenaline molecule. On mixing 1 cc. of adrenaline solution with 2 cc. of 10 % ammonium molybdate solution a yellow colour instantly develops and is permanent for several hours.

It was found that this test, while not so sensitive as the persulphate reaction, could be used in pure solutions for the quantitative estimation of adrenaline down to a dilution of about 1 in 20,000. Unfortunately when applied to concentrated gland extracts it gives very misleading results even when allowance is made for the yellow tint due to the extract alone.

The colour-concentration curve obtained with pure adrenaline is a straight line.

Other points noticed were that alcohol does not affect the reaction, except that too strong a concentration will precipitate the ammonium molybdate and also that the test cannot be applied to urine owing to the presence of other phenolic substances.

Paget [1930] uses this test to differentiate adrenaline and adrenalone, since, although both give an orange colour, the addition of sodium hydroxide discharges this colour to a greenish fluorescence with adrenaline but not with adrenalone. When observed under ultra-violet light adrenaline was found to give a very strong greenish-yellow fluorescence with Paget's test and this constitutes a very sensitive qualitative test for adrenaline, less than 1 in 2,000,000 being detectable. The colours given by adrenaline-free suprarenal extracts show no such fluorescence but the test could not be applied quantitatively without special apparatus.

Folin's test.

Folin's reagent [Folin *et al.*, 1912; 1913] was originally devised for the colorimetric determination of uric acid and many modifications of the reagent and technique employed have been suggested for the determination of uric acid in blood. In this connection Folin and Trimble [1924] attribute the inconsistencies met with by various workers to the presence of varying traces of molybdenum in the sodium tungstate used and describe a method for preparing the uric acid reagent free from such impurity. The sodium tungstate used by the present workers gave no reactions for molybdenum when tested by the xanthate test, and the reagent was therefore made without further treatment as directed by Folin and Trimble.

Although not specific for adrenaline this test is extremely sensitive, being about 10 times more sensitive than the persulphate reaction. The colour produced is a deep blue very similar to that given by uric acid, but it is said to be 3 times as sensitive for adrenaline as for uric acid.

The test has been applied to the estimation of adrenaline by Maiweg [1922] who is also of our opinion that the method gives too high results when applied to suprarenal extracts. Baker and Marrian [1927], in a fuller study of the test, estimated adrenaline in protein-free trichloroacetic acid extracts of suprarenal glands and calculated the experimental error as about 7 %.

Our first attempts to utilise this reaction were carried out as follows. 1 cc. of 1 in 100,000 adrenaline solution was added to 0·5 cc. Folin's reagent and 0·5 cc. saturated Na_2CO_3. The temperature was kept constant at 21°, but even with this precaution results could not be repeated. Elevation of the temperature to 37° resulted in a quicker development of the colour but the same inconsistencies were noticed.

These anomalies were found to be due at least partly to the metal cell used for matching the colours, since, as with the persulphate test, traces of copper dissolved from the metal markedly altered the reaction velocity. This, however, seems to be a peculiarity of the adrenaline test since the colour given by uric acid does not behave in the same way. The matter was not investigated further but the use of an all-glass cell was strictly observed.

Another difficulty encountered was the formation of crystalline precipitates after the mixture had been standing 10 to 15 minutes, the trouble being even worse at 37·5°. Some of the precipitate was removed and analysis showed it to be lithium phosphate which presumably only appears slowly owing to the slow breakdown of the phosphotungstic acid complex. It was found, however, that the use of only half the original quantity of Folin's reagent largely overcame this difficulty, as 45 minutes then elapsed before any precipitate was observed. In spite of these alterations no satisfactory results could be obtained with the test until the importance of the degree of alkalinity of the reaction mixture was discovered. It was found that, owing to the combined buffering action of the carbon dioxide evolved and the phosphates already present, the amount of sodium carbonate added brought the mixture to about p_H 8 only, and it was found necessary to make the reaction at least alkaline to thymolphthalein in order to obtain satisfactory results.

The use of sodium hydroxide as the alkali was therefore adopted, and while this causes the colour to fade rapidly if used in too great excess very consistent results are obtained if the following procedure is adopted. The amount of sodium hydroxide necessary is, of course, partly dependent on the Folin's reagent used and it is suggested that each sample should be titrated to thymolphthalein. In calculating the strength of the alkali to be employed an extra 2 % sodium hydroxide should be added to ensure the required alkalinity of the reaction mixture, *i.e.* if titration shows 0·25 cc. Folin's reagent equivalent to 0·75 cc. of 3 % NaOH, use 0·75 cc. of 5 %. This small excess of alkali is quite safe and does not cause any fading.

We have found that the use of sodium hydroxide as the only alkali constitutes a great advantage both in the determination of adrenaline and of uric acid. Not only does it enable remarkably constant figures to be obtained,

but the colour develops very much more rapidly, the maximum being reached within 3 minutes. The colour is just as stable as when sodium carbonate is used and no troubles are caused by the precipitation of phosphates or by the evolution of carbon dioxide. In view of the fact that many workers find the addition of sodium cyanide advantageous we attribute many of the discrepancies obtained with this test to the question of the p_H, not only in the estimation of adrenaline but also in the estimation of uric acid.

The final modification of this test adopted was as follows. Mix 1 cc. of an adrenaline solution of a concentration between 1 in 100,000 and 1 in 1,000,000 with 0·25 cc. Folin's reagent followed by 0·75 cc. of 5 % NaOH. The colour develops in a few minutes at room temperature and the maximum value is observed in a 1 cm. all-glass cell. The authors found that 0·01 mg. of adrenaline under these conditions gave a colour of 10·6 blue, 1·4 yellow Lovibond tintometer units. With pure solutions of adrenaline a straight line is obtained for the colour-concentration curve.

The potassium persulphate reaction.

Ewins [1910] described this test as the most sensitive reaction for adrenaline (giving a lower limit of 1 in 5,000,000). He also examined the effect of persulphate on various bases allied to adrenaline and found that the oxidation products such as aminoacetocatechol and its derivatives did not give the reaction. When applied to suprarenal extracts which were free from adrenaline we found that this test gave no colour and it was decided to investigate it more thoroughly in order to establish it on a quantitative basis.

Preliminary trials soon showed that Ewins had rather overestimated the sensitivity and that for quantitative measurement the adrenaline content should be not less than 1:100,000. The test was carried out by mixing 1 cc. of the solution under test with 1 cc. of 0·2 % $K_2S_2O_8$. The concentration of the adrenaline solution was kept constant at 1:10,000 and was made by diluting Liq. Adrenalin. Hydrochlor. B.P. 1914.

Effect of temperature.

The effect of variation of temperature was first studied. The colour was measured in a 1 cm. cell in the Lovibond tintometer at intervals, and the intensity plotted against the time (see Figs. 1 and 2), at temperatures of 18° and 37·5° respectively. It will be noticed that the maximum colour developed is slightly greater at 37·5°, being about 7·6 red, 3·4 yellow units and that the time required to reach this is much less at this temperature, actually about 25 minutes instead of the 55 minutes required at 18°. Further, the colour is stable over a longer period at 37·5°. The red tint is about double the yellow, but both tints are produced at about the same rate although the yellow starts to fade slightly before the red. When the red does fade, however, it does so much more quickly, especially at 37·5°.

Further experiments showed that at 0° the colour hardly develops at all, whilst at higher temperatures, 60° and 100°, although the colour appears

rapidly, it also fades very rapidly. Attempts to prevent this rapid fading by sudden cooling in a freezing mixture were not very successful and in no case was the colour obtained as high as that at 37·5°.

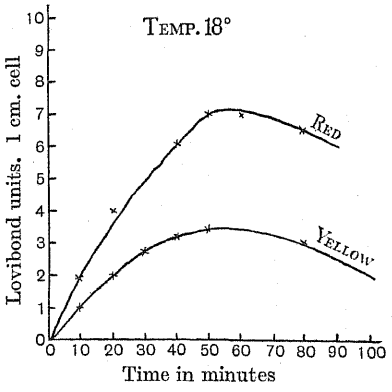


Fig. 1.

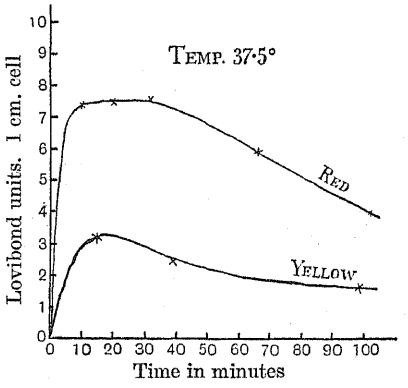


Fig. 2.

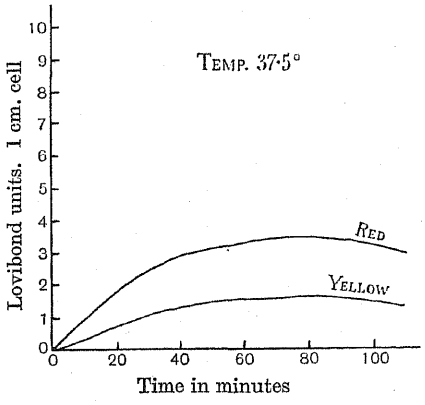


Fig. 3.

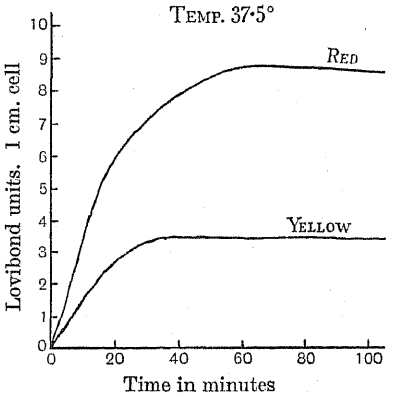


Fig. 4.

Fig. 1. 1 cc. 1 : 10,000 adrenaline (HCl). 1 cc. 0·2 % potassium persulphate.

Fig. 2. 1 cc. 1 : 10,000 adrenaline (HCl). 1 cc. 0·2 % potassium persulphate.

Fig. 3. 1 cc. 1 : 10,000 adrenaline (H_2SO_4) solution. 1 cc. 0·2 % potassium persulphate.

Fig. 4. 1 cc. 1 : 10,000 adrenaline (H_2SO_4) solution. 1 cc. 0·2 % potassium persulphate, 1·0 % sodium chloride.

Effect of chlorides.

When an attempt was made to confirm the above results, using a freshly made solution of adrenaline sulphate, very much lower figures were recorded. Thus, at 18° the colour development was almost nil, although it was higher at 37·5° (see Fig. 3). The original figures were confirmed, however, by diluting some freshly made Liq. Adrenalin. Hydrochlor. B.P. 1914 and it was obvious therefore that either the chloroform or the sodium chloride present in the B.P. liquor, or perhaps both, was responsible for the increased colour.

Experiment showed that the presence of chloride was the important factor and the effect of the addition of 1·0 % of sodium chloride to the reagent, using a 1 in 10,000 adrenaline sulphate solution, is shown in Fig. 4. This curve showed an improvement on all the previous work since not only was the maximum colour higher but it was established in a shorter time. Further, the colour was stable over a very much longer period. This stabilising effect of chlorides has been utilised in all the subsequent work, 1·0 % of sodium chloride being added to the reagent.

Effect of calcium chloride.

It has been noted by various workers that very dilute solutions of adrenaline in tap water turn pink much more rapidly than similar solutions in distilled water.

Two explanations suggested were:

(*a*) the chemical effect of dissolved substances in tap water;

(*b*) the buffering effect of tap water.

At first the conclusion was reached that the calcium present in the tap water was responsible for this phenomenon since the addition of 0·2 % $CaCl_2$ to the persulphate reagent resulted at 18° in a quicker establishment of the maximum colour. The only other observable effects were that slightly less yellow was produced and that the stabilising effect of the sodium chloride largely disappeared at 37·5° although it was retained at room temperature.

A modification of the test using a reagent containing 0·2 % $CaCl_2$, 1·0 % NaCl, 0·2 % $K_2S_2O_8$ was tried, but it was discovered that the reduction of the calcium chloride from 0·2 % to 5 parts per million produced no different results. We had to assume, therefore, that the effect of calcium chloride was either catalytic in nature or that it was entirely fortuitous and our attention was turned to a consideration of the p_H values during the reaction.

Effect of p_H *value.*

It is well known that the change of colour of adrenaline solutions brought about by oxidising agents is less rapid in acid solution than in neutral solution and more rapid in alkaline solution but we have not been able to find any record of an investigation of the effect of p_H upon the maximum colour produced by any given quantity of adrenaline or upon the time necessary for the production of that maximum coloration. This is a factor of great importance if the test is to be employed from the quantitative point of view, for variation of the p_H value of the reaction mixture was found to produce widely differing results.

Normally the p_H of the mixture was about 4·6, and the maximum colour was obtained in 30 minutes, but after the addition of acid to p_H 2 the maximum colour was not reached even after 7 hours. Attempts to determine the optimum p_H value for the reaction were at first unsuccessful, but this was traced to an increase in the p_H of the mixture as the reaction proceeded. It was therefore

necessary to incorporate a buffer in the reagent and several were tried, but those containing organic acids, especially citric acid, were excluded. The ordinary phosphate buffers were adopted, and the p_H values of the reaction mixtures were determined electrometrically using the quinhydrone electrode. Caution was necessary since the presence of an oxidising agent resulted in the cell becoming more acid on standing, and readings had to be taken immediately after adding the quinhydrone in order to obtain consistent results.

The time required to establish the maximum colour at 22° was plotted against the p_H value, keeping the adrenaline constant (1 in 10,000) and this curve is shown in Fig. 5. It clearly shows the optimum p_H value to be at p_H 5·5 and that if the mixture is either more acid or alkaline than this a very much longer time is required to establish the maximum colour.

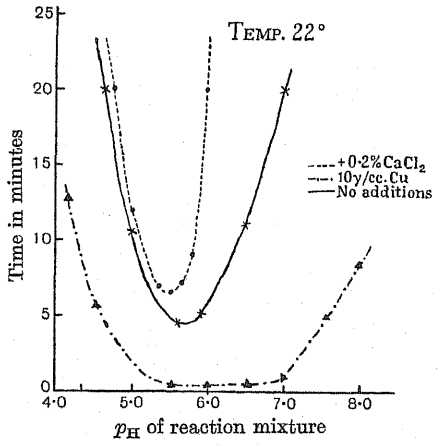


Fig. 5.

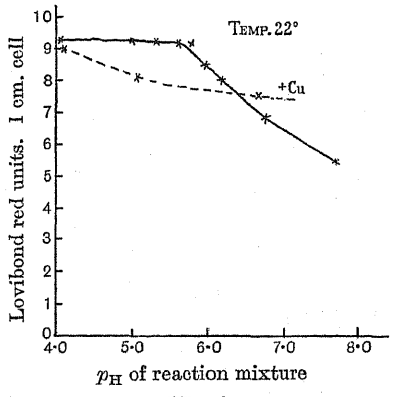


Fig. 6.

Fig. 5. Relation between time required to establish maximum colour and p_H of mixture (adrenaline 1 : 10,000). All in phosphate buffer.

Fig. 6. Effect of p_H on maximum colour. 1 cc. 1 : 10,000 adrenaline (HCl) solution. 1 cc. 0·2 % $K_2S_2O_8$, 1·0 % NaCl in phosphate buffer.

In Fig. 6 the maximum colour is plotted against the p_H value. It should be noted that only in solutions more alkaline than p_H 5·8 is there any reduction of the maximum colour so that fortunately in all our previous work, where the p_H happened to fall about 4·6, only the time required and not the actual maximum colour was liable to variation.

Also in Fig. 5 is given a similar curve in which 0·2 % $CaCl_2$ was present. This curve, although almost the same as that obtained in the absence of calcium, rises very sharply at p_H 6·0, and 24 hours are required at p_H 6·1 for the development of the maximum colour. This point coincides with the appearance of a precipitate of calcium phosphate. Thus it is obvious that no catalytic effect is associated with the calcium ion and the use of calcium chloride in the reagent was discontinued.

Catalytic effect of copper.

A chance observation emphasised the fact that copper will catalyse reactions of this kind. It was found that the colour developed more quickly in a metal cell than in an all-glass cell and this was found to be due to the dissolution of minute amounts of copper from the imperfectly plated framework of the cell. The curves obtained in the presence of 10 parts per million of copper (as sulphate) are shown in Figs. 5 and 6, using as reagent 0·2 % $K_2S_2O_8$ and 1·0 % NaCl in phosphate buffer solution. The catalytic effect results in the maximum colour being developed in less than half a minute over a wider p_H range (5 to 6). Fading is, however, much more marked although it does not occur in solutions more acid than p_H 4. This catalytic effect is intimately connected with the stability of the persulphate reagent, which probably functions in a similar manner to hydrogen peroxide. Other heavy metals such as mercury, cobalt, nickel and manganese also show some catalytic effect but not to the same degree as copper.

Iron on the other hand shows a slight inhibitory effect.

Colour-concentration curve.

As a result of the above work the following reagent was adopted and should be made with A.R. materials: 0·2 % $K_2S_2O_8$, 1·0 % NaCl, 0·239 % Na_2HPO_4, $12H_2O$, 0·937 % NaH_2PO_4, $2H_2O$. This solution should have p_H 5·5, and is stable if kept away from the light and in a cool place, but caution must be observed since when kept for some weeks at room temperatures the reagent tends to become more acid in spite of the buffer present, presumably owing to the release of sulphuric acid by the decomposition of the persulphate.

The colour-concentration curve for pure adrenaline (using the above reagent) is given in Fig. 7. Both the red and yellow tints give straight lines and it should be observed that both curves meet the horizontal axis at a point

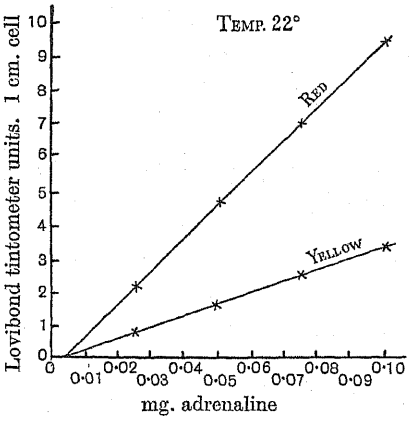


Fig. 7. Colour-concentration curve persulphate test. 1 cc. adrenaline solution (HCl). 1 cc. 0·2 % $K_2S_2O_8$, 1 % NaCl in phosphate buffer at p_H 5·5.

corresponding to 0·005 mg. of adrenaline. This point represents the limit of sensitivity of the test.

Application of the test to gland extracts.

Having thus determined the most favourable conditions for carrying out the test in pure solutions the method was applied to gland extracts. It was found that the colour did not develop so rapidly as in pure solutions, requiring about 30 minutes at 22°, probably because of the greater amount of easily oxidisable organic material in such an extract. The addition of a trace of copper sulphate enables the test to be carried out in less than 5 minutes but the results are less accurate and should not be used except as a preliminary indication.

The method recommended for gland extracts is as follows. Adjust the p_H of the extract to 5·4 (methyl red, external indicator). Then to 1 cc. add 1 cc. of the potassium persulphate reagent and immediately measure the colour (red units) in a tintometer using a 1 cm. all-glass cell. Then place the cell in the thermostat at 22° and read the colour again after 30 minutes. The increase of the red units is then compared with that given by a standard solution of adrenaline treated in a similar manner. The method can obviously be modified for comparison in a colorimeter.

The test is seriously interfered with by alcohol of which even 1·0 % affects the time required to produce the maximum colour. It is recommended that alcoholic solutions should be evaporated *in vacuo* making sure that the solution is more acid than p_H 5 and not allowing the temperature to rise above 30°. When free from alcohol, the residue is made up to the original volume with distilled water, and then tested as usual. No loss of adrenaline occurs under these conditions.

Comparison of tests on other substances.

The three tests found suitable for estimating adrenaline in pure solutions, *viz.* molybdate, persulphate and Folin's tests, were applied to a variety of organic substances which might cause interference and the results are given in Table I.

It will be noticed that Folin's test is not specific for adrenaline and, besides uric acid, most easily oxidisable substances give some blue colour, especially the aromatic polyhydroxy-compounds.

The molybdate test is, however, much better, tannic acid, and pyrogallol being the only other substances tried which gave the reaction. This, of course, is to be expected as they contain the catechol grouping. The fact that this test indicates the catechol group also means that substances closely allied to adrenaline, but without its pressor activity, may be the source of error encountered on applying this test to suprarenal extracts.

The persulphate test is the most specific test for adrenaline and (of the substances examined) gives a positive test only with metol and *p*-anisidine

Table I.

Test substance	Ammonium molybdate	Potassium persulphate	Folin's
Adrenaline	Orange-yellow	Red	Deep blue
Histamine	Nil	Nil	Nil
Liquid extract of ergot	Very slight pink	Nil	Prussian blue
Tyramine	Nil	Nil	Nil
Metol. OH⟨⟩$NHCH_3$	Nil	Red (on boiling only)	Intense blue
Salicylic acid	Yellow on warming; discharged by NaOH	Slight yellow	Nil
Tannic acid	Reddish orange; no u.v. fluorescence	Slight yellow	Intense blue
Tyrosine	Nil	Nil	Very slight blue
Trypsin	Very slight yellow	Nil	Blue
Vanillin	Nil	Nil	Very slight blue
Pyrogallol	Orange (as tannic acid)	Orange-brown on heating	Intense blue
o-Cresol	Very slight yellow on heating	Yellow on heating	Very slight blue
Salicylic aldehyde	Slight yellow on heating	Nil	Green-yellow precipitate on heating
Uric acid	Nil	Nil	Intense blue
Meat extract	Nil	Nil	Blue
Urine	Nil	Nil	Intense blue
p-Anisidine	Nil	Red on boiling	Nil
Phenacetin	Nil	Nil	Nil
Aniline	Nil	Slight purple	Nil

and then only on boiling. This test seems to depend on the presence of a primary or secondary amino-group in the side-chain and an —OH group in the *para*-position in the benzene ring. If, however, in addition to these groups a ketone group is present in the side-chain no reaction is obtained.

Ewins [1910] found that the following bases allied to adrenaline will give a positive persulphate reaction: arterenol (dihydroxyphenylethanolamine), dihydroxyphenylethylamine and epinine (dihydroxyphenylethylmethylamine), whereas the following will give no colour: homorenon (ethylaminoacetocatechol), methylaminoacetocatechol, ephedrine.

It should be noted that although some of these bases give the persulphate reaction, those which do so possess marked physiological action very similar to, but weaker than that produced by adrenaline. The corresponding ketonic oxidation products on the other hand do not give the colour test and do not generally possess physiological action much greater than that of catechol and its simple derivatives.

Comparison of colour tests with the biological method.

A comparison of the colour tests with biological results was made in order to prove definitely the impressions gained from previous work, *viz.* that Folin's and the molybdate tests were unreliable for the estimation of adrenaline in concentrated suprarenal extracts.

A sample of suprarenal cortex extract was taken and known quantities of standard adrenaline solution were added giving four extracts with upper and

lower limits of adrenaline at about 1 in 5000 and 1 in 100,000 respectively. The adrenaline was then determined colorimetrically in each extract by the ammonium molybdate, potassium persulphate and Folin's tests. Samples were assayed biologically at the same time.

Table II summarises the results obtained, the corresponding curves being shown in Fig. 8.

Table II. *Adrenaline in suprarenal extracts.*

Results as parts per 10,000.

	Extract "A"	"B"	"C"	"D"
Adrenaline actually present assuming "A" to contain 0·1 parts per 10,000	—	0·278	1·01	1·91
Persulphate test	<0·1	0·29	1·01	1·98
Folin's test	0·61	—	2·02	3·04
Molybdate test	1·5	3·9	5·4	6·7
Biological test	0·11	0·23	0·78	1·7

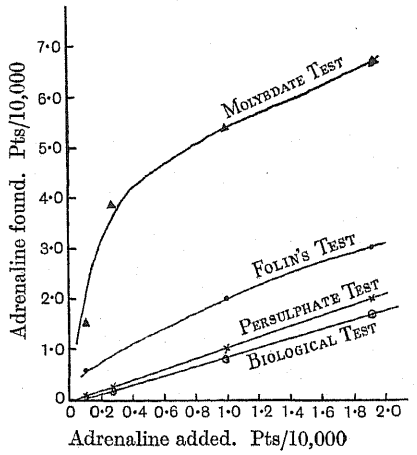


Fig. 8. Comparison of tests on gland extracts.

It will be seen that neither Folin's test nor the molybdate test gives reasonably correct results with gland extracts. The error of Folin's test is less than that of the molybdate test and in some cases might be very much less than that shown in Table II, since some samples of suprarenal extract gave much less colour with Folin's reagent, although of the same adrenaline content. The increase from sample "C" to "D" shown by Folin's test is in good agreement with that expected. Since such abnormal samples are always liable to occur, the use of Folin's test for quantitative work must be abandoned in spite of its great sensitivity, but negative results can be safely interpreted as indicating absence of adrenaline.

The results given by the persulphate test are as close as can be expected to the biological figures and are extremely close to the values anticipated, the

increases from samples "B" to "C" and from "C" to "D" being exactly the amounts actually added.

Attempts were made to use the persulphate test at dilutions below 1 in 100,000, and it was found possible by utilising the Klett colorimeter to carry out determinations with a degree of accuracy comparable with that of the biological method.

The estimation of adrenaline in glands.

It must be remembered that the suprarenal gland extracts described in this paper are of high concentration and naturally give the extremes of divergence of the various tests for adrenaline. In the quantitative determination of adrenaline in the suprarenal glands themselves, we obtained satisfactory results by using the protein-free trichloroacetic acid extracts described by Baker and Marrian [1927], who make use of Folin's reagent.

It is important, however, not to neutralise the filtrate before adding the reagents (Folin's or persulphate). Adrenaline is quite stable in trichloroacetic acid but if the solution is neutralised with sodium hydroxide, as the p_H approaches a value of about 3, a colour reaction takes place, a red colour forming which quickly fades to a fluorescent yellow. This results in the destruction of a large part of the adrenaline, but can be avoided by adding first the reagent and then afterwards the requisite quantity of alkali, allowing of course for the effect of the volume added on the dilution of the colour. Ox suprarenal gland by the persulphate test applied in this way showed an average adrenaline content of 3·3 mg./g. fresh gland, with 2 mg./g. in the cortex and 9 mg./g. in the medulla. The filtrates obtained from the trichloroacetic acid precipitation of defibrinated blood showed no adrenaline by the persulphate test. Addition of known amounts of adrenaline to the blood and subsequent precipitation showed that the persulphate test gave correct results (2 % loss) but the Folin's test gave results which were 25 % too high.

Summary.

1. The need for a rapid and accurate quantitative test for adrenaline in highly concentrated suprarenal gland extracts led to the investigation of many of the colour tests described in the literature. Some of these are unsatisfactory even from a qualitative point of view; others are non-specific. The latter criticism applies to the ferric chloride, ammonium molybdate and phosphotungstic acid (Folin's) tests, although the latter is extremely sensitive, and gives good results with comparatively pure adrenaline solutions if applied in accordance with the slightly modified technique described in this communication.

2. The potassium persulphate test has been investigated more thoroughly and although it is not as sensitive as Folin's test it gives results comparable with those obtained by the biological test when the necessary conditions, including control of temperature and p_H, are observed.

3. Preliminary work upon the application of the persulphate test to the determination of adrenaline in the suprarenal glands themselves indicates that it is of sufficient accuracy for this and similar purposes.

We are indebted to Mr F. Wokes for carrying out the biological tests.

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CCLIII. THE APPLICATION OF PHOSPHO-18-TUNGSTIC ACID (FOLIN'S REAGENT) TO THE COLORIMETRIC DETERMINATION OF CYSTEINE, CYSTINE AND RELATED SUBSTANCES.

I. THE REDUCTION OF PHOSPHO-18-TUNGSTIC ACID BY VARIOUS SUBSTANCES.

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Of the phosphomolybdates and phosphotungstates those containing eighteen atoms of the heavy metal to every P_2O_5, members of the 1–18 series in our present nomenclature, have been exploited most in colorimetric estimations on account of the ease with which their pale-coloured solutions may be reduced by certain substances to give intense blue, greenish, or reddish tints. Some use has lately been made of the corresponding arsenic compounds.

Wu [1920] arranges the 1–18 and 1–24 phospho-molybdates and -tungstates in order of increasing sensitiveness to reduction, as follows: phospho-24-tungstic acid, B-phospho-18-tungstic acid, A-phospho-18-tungstic acid, phospho-24-molybdic acid, and phospho-18-molybdic acid. He further states that acids of the 1–17 and 1–22 series are quite inert. Salts of the 1–17 and 1–22 series are formed when those of the 1–18 and 1–24 series respectively are treated with excess of alkali hydroxide or carbonate. Salts of phospho-17-tungstic acid are thus precipitated as small white crystals difficultly soluble in large excess of alkali. These salts, together with others containing much more phosphorus which are supposed to exist, are said to regenerate 1–18 and 1–24 acids exclusively on treatment with mineral acids.

Wu concluded that two forms, A and B, of phospho-18-tungstic acid exist, and that his B form may be identified with the 1–18 acid of Kehrmann [1887], who was the first to prepare such a compound. He notes however that Kehrmann probably prepared both forms. The A and B forms are said to yield the same 1–17 acid on treatment with alkali, and the 1–17 acid regenerates exclusively the B 1–18 acid on treatment with mineral acids.

Some doubt has been thrown upon the existence of the A form by Folin and Marenzi [1929], who suggest the presence of molybdenum impurity in Wu's sodium tungstate and that the alleged A acid may have been a molybdo-tungsten complex.

Folin and Looney [1922] made use of the Folin and Denis [1912] uric acid reagent to determine the cystine content of protein hydrolysates. The uric acid reagent is essentially phospho-18-tungstic acid prepared by treating sodium tungstate with phosphoric acid, and if Wu's findings are correct one would expect it to contain both the A and B forms. The method is based upon the observation (apparently unpublished) of T. Zucker, that cystine *plus* sodium sulphite gives colours with the uric acid and phenol reagents, whereas cystine alone does not, and sulphite alone develops a comparatively feeble colour. Heffter [1907] had discovered that cystine may be reduced by sulphite to give cysteine.

Since 1922 many modifications of the Folin-Looney method have appeared. Hunter and Eagles [1927] warned against having standard and unknown at even slightly different degrees of alkalinity. Folin and Marenzi [1929], following the lead of Folin and Trimble [1924], carefully removed molybdenum from the reagent to avoid reactions with tyrosine, tryptophan and phenolic bodies generally. They also improved the technique in some directions. Rimington [1930], working upon an observation made by Folin, added urea to prevent precipitation of the 1–17 tungstate; an event that frequently ruins estimations under the Folin-Looney and Folin-Marenzi procedures. Folin and Marenzi themselves had tried to overcome this difficulty by adding large amounts of lithium salt to the reacting mixture. In their mixtures the solubility of lithium carbonate is so grossly exceeded that it is surprising its precipitation does not occur more frequently than it actually does. Tompsett [1931] reduced the alkalinity of the reacting mixture and thereby avoided precipitation of the 1–17 tungstate, reduced the effect of slight changes in alkalinity upon the colour developed, and greatly reduced interference by uric acid. Most of the solutions (*e.g.* hydrolysates of tissues) which have to be analysed for cystine contain other substances which contribute to the coloration developed. Uric acid gives much colour in both the Folin-Looney and Folin-Marenzi procedures in absence of sulphite, and more in presence of it. Those soluble products arising from the digestion of carbohydrate with mineral acid at the boiling point also reduce the reagent. It has become customary to subtract the blank obtained in absence of sulphite from the colour obtained in presence of sulphite, and to call the nett result "cystine." Inasmuch as the coloration developed by extraneous substances may be increased when sulphite is present, and perhaps reduced by the very presence of cystine, this procedure is unjustified. Furthermore, cysteine reduces the reagent in absence of sulphite, and this seems to have been neglected in nearly all interpretations of the results, the blank obtained in absence of sulphite being calculated nearly always as "uric acid."

In his study of the alkaline decomposition of cystine, Andrews [1928] used the Folin-Looney procedure for the estimation of both cysteine and cystine. With some concern he noted that the colour developed in presence of sulphite sometimes exceeded that which would have been obtained if none of the cystine had been destroyed, and ascribed this remarkable result to the presence of

some unknown strongly reducing substance produced in the treatment of cystine with alkali. His expectations were really based upon the accepted belief that sulphite reduces cystine to cysteine under the conditions of the cystine estimation. The fate of the cysteine during its oxidation by the reagent seems not to have interested anybody.

There is reason to believe that any sufficiently soluble sulphhydryl compound will reduce the reagent, and the same may be said of any disulphide which will react with sulphite to form a sulphhydryl compound. It is clear then that the reaction with phospho-18-tungstic acid is not only unspecific for cysteine among the sulphhydryl compounds, but is further unspecific for sulphhydryl compounds themselves. This lack of specificity has led many to abandon phosphotungstic acid in favour of a far more specific colour reaction discovered by Sullivan [1926], and, in turn, the enormous difficulties attending the application of Sullivan's reaction to quantitative analyses have thrown the list of estimations in the literature into a rather chaotic state.

The present paper constitutes an analysis of the phospho-18-tungstate colour reactions, and includes among other things, investigations of the nature of the reaction between cysteine and the reagent, the reactions between cystine and sulphite, and the degree to which the reagent is reduced by substances other than cysteine but likely to occur in a mixture to be analysed for cysteine or cystine.

Experimental.

The phospho-18-tungstic acid was prepared free from molybdenum according to the method of Folin and Marenzi [1929]. Some of the reducing substances examined were carefully prepared in this laboratory. I am indebted to Mr H. R. Marston of this Division for the supply of cystine.

The coloration developed by a reducer may vary with the time allowed for the reaction, the concentration and amounts of the reacting materials, the p_H of the reacting mixture, salt concentration and type, the order in which the materials are added, and sometimes with small amounts of substances which may react with a particular reducer under examination. With the more powerful reducers the effect of temperature seems to be almost nil.

Except in alkaline solutions, the order of adding the different materials has but little effect upon the colour developed in 5 minutes. In alkaline regions slightly greater colorations result if the reagent is first made alkaline and the reducers are then added, than if the reagent and reducer are first mixed and then made alkaline. This effect increases slightly with the time for which the alkaline reagent is allowed to stand, provided this is limited to a few minutes. Solutions containing cystine *plus* sulphite, cysteine, or ferrous salt must never be made alkaline before the reagent is added, or there will be a decided loss in coloration developed (*cf.* the Folin-Looney and Folin-Marenzi procedures for the estimation of cystine). Na_2S as reducer cannot be added to an acid solution and made alkaline afterwards. Such procedure would result in a loss of H_2S.

Fig. 1 expresses graphically the variation with p_H of the blue coloration developed by some of the reducers and mixtures, under conditions to be described. Different amounts of reagent were used in obtaining different curves, but for each curve the only important independent variable was the p_H. The free phosphate in the reagent acted as a buffer in certain regions but most of the buffering was done by acetate, citrate, and borate mixtures added for that purpose. In the more alkaline regions sodium carbonate was used to "neutralise" the reagent after the reducer had been added, and in the more acid ones sodium acetate was used. Temperature varied very little from 25°.

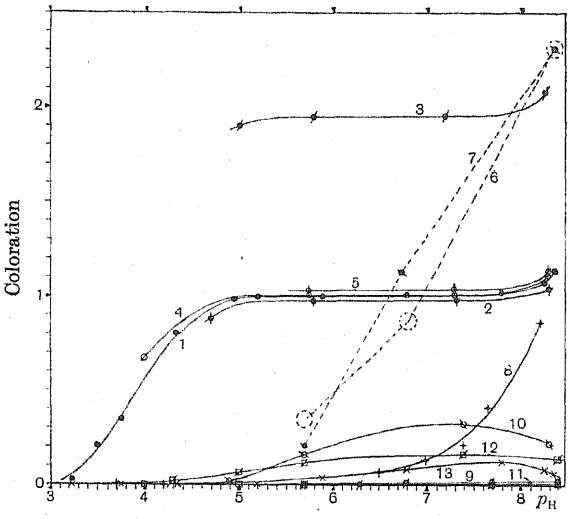


Fig. 1. 1, cystine + sulphite; 2, cysteine; 3, cysteine + sulphite; 4, Fe++; 5, Fe++ + sulphite; 6, Na_2S; 7, Na_2S + sulphite; 8, uric acid; 9, furfuraldehyde; 10, furfuraldehyde + sulphite; 11, laevulinic acid; 12, laevulic acid + sulphite; 13, sulphite.

Short of recording in a lengthy table the actual amounts of carbonate, citrate, acetate, *etc.*, used it would be impossible to state the actual salt concentrations, but it is believed that the reduction in the activity of the water in the final diluted solutions would correspond approximately with that observed in a 0·4 *M* NaCl solution. In so far as salt type and concentration may greatly influence the activities of the species taking part in the colour reactions, the point is however not without importance. Two sets of solutions were used in obtaining each curve. They differed only in that the members of the set used in estimating the coloration received reducing substance whilst the corresponding members of the other set, used in p_H determination, did not. p_H was determined colorimetrically with the aid of a wide range of indicators and recorded values may be in error by 0·1 unit or more in some instances.

The colour reactions were carried out in 100 cc. standard flasks. Colour development was allowed to proceed for 5–6 minutes in a volume not exceeding 30 cc. and not less than 15 cc., the volume being kept constant for any one

substance under investigation whatever the p_H or buffers employed. After this interval had elapsed the solutions were diluted to the mark with pure water and within 5 to 15 minutes were compared with some arbitrary and constant standard. It was found necessary to have some citrate present in alkaline solutions containing iron, tin, *etc.* to avoid formation of precipitates. Lithium sulphate was never added. The p_H beyond which one cannot proceed without precipitating the 1–17 tungstate is 8·5. The curves are for colorations obtained with:

1. 2 mg. (1/120 millimol.) of cystine *plus* 1 millimol. of Na_2SO_3 with 5 cc. of reagent.
2. 2·017 mg. (1/60 millimol.) of cysteine alone with 2 cc. of reagent.
3. 2·017 mg. of cysteine *plus* 1 millimol. of sulphite with 2 cc. of reagent.
4. 0·93 mg. (1/60 millimol.) of Fe^{++} alone with 2 cc. of reagent.
5. 0·93 mg. of Fe^{++} *plus* 1 millimol. of sulphite with 2 cc. of reagent.
6. 1·30 mg. (1/60 millimol.) of Na_2S alone with 10 cc. of reagent.
7. 1·30 mg. of Na_2S *plus* 1 millimol. of sulphite with 10 cc. of reagent.
8. 2 mg. (1/64 millimol.) of uric acid alone with 5 cc. of reagent.
9. 20 mg. (1/4·85 millimol.) of furfuraldehyde alone with 2 cc. of reagent.
10. 2 mg. of furfuraldehyde *plus* 1 millimol. of sulphite with 2 cc. of reagent.
11. 10 mg. (1/11·6 millimol.) of laevulic acid alone with 2 cc. of reagent.
12. 10 mg. of laevulic acid *plus* 1 millimol. of sulphite with 2 cc. of reagent.
13. 1 millimol. of Na_2SO_3 with 5 cc. of reagent.

In order that the figure might not be unduly complicated, many of the data have been left out of it, and some of these will be recorded below.

When carefully purified and fresh, pyruvic acid (10 mg.) does not reduce the reagent at any p_H. In acid regions the coloration developed in presence of sulphite is practically that of the sulphite itself, but in more alkaline regions the coloration is somewhat greater than that due to the sulphite alone. Pyruvic acid polymerises on standing and old, impure samples may reduce the reagent.

Creatinine only slightly reduces the reagent, the least reduction being observed in acid solutions. Quinol reduces the reagent strongly in alkaline regions and fairly well in acid solutions. Phenylhydrazine and "metol" are powerful reducers. Cuprous chloride reduces the reagent after the manner of ferrous salt. Ferric chloride *plus* sulphite gives a colour corresponding with that of the sulphite alone. Stannous chloride alone gives a slight colour in acid solution but a stronger one in alkaline. With sulphite the colour developed is much greater than mere addition would allow, and the effect is therefore reminiscent of that observed with furfuraldehyde or laevulic acid. Ethyl sulphide reduces only slightly in acid solution and rather more strongly in alkaline. It is very sparingly soluble. The addition of sulphite intensifies the colour somewhat. It is probable that the sample contained a trace of mercaptan or of a disulphide, or both. Oxalates, malates, and tartrates have no effect upon the reagent. Tetrathionates seem to be without effect.

Stability.

Colorations developed by cysteine, cystine *plus* sulphite, cysteine *plus* sulphite, ferrous salt, and ferrous salt *plus* sulphite are extremely stable in the region p_H 5·0–6·5. As a rule they increase by 1 or 2 % in the course of several hours and then begin to fall. Using 5 cc. of reagent at p_H 5·7, the coloration with 2 mg. of cystine *plus* 1 millimol. of Na_2SO_3 is reduced by only 5 % in 18 hours, whereas at p_H 8·35 the loss may be 30 %. After 48 hours the losses might be 20 and 50 % respectively. Below p_H 0·4 the development of colour by cystine *plus* sulphite is extremely slow and the depth of coloration increases considerably during several hours. The coloration developed by cysteine alone or ferrous salt, on the other hand, comes up rapidly in acid mixtures, but in the case of ferrous salt, with or without sulphite, the solutions fade during dilution and for some time thereafter. In acid solutions, colorations given by Na_2S increase considerably with time.

Colorations developed by sulphite alone, laevulic acid with and without sulphite and furfuraldehyde with and without sulphite increase relatively much more in the first few hours and fade much more slowly.

The coloration developed by quinol is curious in that it fades extremely rapidly. At p_H 6·5 for example, the loss may be 50 % within 30 minutes.

The coloration developed by uric acid alone progressively increases in acid solution during 18 hours and longer, whereas in alkaline solution it fades much more rapidly than in the case of cystine *plus* sulphite. Besides greatly increasing the depth of coloration with uric acid, sulphite improves the stability in alkaline solution.

The effects of varying the amounts of reagent, etc.

A series of cystine *plus* sulphite colorations developed by only 2 cc. of reagent, yielded a curve rather less than 2 % below the one in Fig. 1 over the range p_H 5·0–7·5. Below p_H 5 the curve approached the abscissa more steeply than the one in the figure and above p_H 7·5 the curve rose less steeply, thereby following more closely the curves for cysteine and ferrous salt (both also obtained with 2 cc. of reagent) in this region and down to p_H 5. The reverse variations from the curve in the figure were observed when 8 cc. of reagent were employed. The amount of sulphite could be doubled or halved without affecting the coloration developed with cystine by more than 2 %, provided the reactions were carried out above about p_H 5·2 and that sufficient time, the usual 5–6 minutes, was allowed for colour development. The rate of colour development however was greatly influenced by varying the quantity of sulphite, rate and quantity of sulphite decreasing together, and more so the lower the p_H. Below p_H 5 the curve became steeper as the quantity of sulphite employed was decreased. The shape of the curve below p_H 5 therefore depends largely upon the amounts of sulphite and reagent employed and the

time allowed for the reaction, as it tends to flatten out if the 5–6 minute interval is increased.

The colorations developed by cysteine and ferrous salt do not vary much with the amount of reagent, the effects at each end of the curves being less pronounced than they are with cystine *plus* sulphite.

On the other hand the coloration with sulphite alone increases with increasing amounts of reagent but not linearly. These remarks apply also to furfuraldehyde and laevulic acid, alone and with sulphite, but for uric acid alone the depth of coloration is nearly proportional to the amount of reagent over the entire p_H range.

The coloration developed by Na_2S in the 5–6 minute time interval increases with the amount of reagent when only a few cc. are used. With 5 or 10 cc. there is not much difference in the amount of colour developed at high p_H values.

The rate of colour development decreases as the volume of the reacting mixture is increased. With large volumes, full colour development may be attained with cysteine or ferrous salt in 5 or 6 minutes, but with cystine *plus* sulphite or with Na_2S this may not be so, particularly in acid regions.

Proportionality.

The depth of coloration developed by cysteine, cystine *plus* sulphite, ferrous salt, and Na_2S, the last subject to certain restrictions in the p_H range, is strictly proportional to the amount of reducer employed within the usual limits imposed by the nature of colorimetry, provided that the chosen p_H be maintained and that sufficient reagent be present. Thus in presence of 2 cc. of reagent, 4 mg. of cystine *plus* sulphite give approximately 3·95 times as great a coloration as is developed by 1 mg. of cystine *plus* sulphite at any fixed p_H in the range 5·0–8·4.

At any chosen p_H cysteine *plus* sulphite gives almost exactly (to within 1 %) twice the coloration developed by the cysteine alone. The curves showing this relationship may be seen in Fig. 1.

At any chosen p_H the depth of coloration developed by uric acid is far from proportional to the amount present, reagent concentration remaining the same. Thus with 2 cc. of reagent at p_H 8·4, 2 mg. of uric acid give a coloration only 20 % greater than does 1 mg. If sulphite is present and/or the amount of reagent be increased, a deeper coloration is developed and the poor proportionality is improved. Thus under the same conditions as before, but with 1 millimol. of sulphite present, 2 mg. of uric acid give a 40 % greater coloration than does 1 mg.

Other variables remaining the same, the depth of coloration developed by sulphite alone is approximately proportional to the amount of sulphite present.

Furfuraldehyde alone and laevulic acid alone, give colorations varying more or less directly with the amounts present when other variables are unaltered. The colorations developed in presence of a fixed amount of sulphite however are by no means proportional to the amounts present. There

appears to be no maximum with laevulic acid, but with 2 cc. of reagent at p_H 5·7 and in presence of 1 millimol. of sulphite, 3 mg. of furfuraldehyde give a greater coloration than any other amount. In presence of 1 millimol. of sulphite at p_H 5·7, 0·25 and 0·5 millimol. of $SnCl_2$ develop about the same degree of coloration.

Inhibition and enhancement of colour development.

Sufficient acetone, if added before reagent and sulphite, will completely inhibit the production of colour by cystine for some distance on either side of p_H 5·7. 2 mg. of cystine *plus* 1 millimol. of purified acetone-bisulphite compound will produce at p_H 5·7 about 80 % of the coloration obtained by using sulphite instead of acetone-bisulphite, and 1 millimol. of acetone-bisulphite will give only a fraction of the coloration developed by 1 millimol. of sulphite alone.

In presence of sufficient furfuraldehyde (*e.g.* 150 mg.) cystine *plus* sulphite fails to reduce the reagent appreciably.

When a fairly large amount (1 or 2 millimol.) of cadmium chloride is added to a solution containing cystine and followed by sulphite and reagent, the coloration develops extraordinarily slowly so that at the end of 5 minutes only 60 % of the full colour value may be attained at p_H 5·7. $CdCl_2$ also reduces the depth of coloration developed by laevulic acid *plus* sulphite and by furfuraldehyde *plus* sulphite.

Zinc chloride in quantity (4 millimol.) will reduce the coloration developed by cystine *plus* sulphite at p_H 5·7 by several %. 2 millimol. of $ZnCl_2$ do not seem to alter the coloration given by sulphite alone. The coloration developed by cysteine is very slightly decreased and that by ferrous salt not at all. 2 millimol. of $ZnCl_2$ will reduce the coloration given by 10 mg. of laevulic acid *plus* sulphite by as much as 35 %. The coloration given by furfuraldehyde *plus* sulphite is also greatly reduced.

Mercuric chloride does not alter the coloration given by sulphite alone, but in the presence of even such a small amount as 0·1 millimol. of $HgCl_2$, cysteine alone fails to develop a trace of colour with the reagent at p_H 5·7, and cystine *plus* sulphite yields only the colour which would be expected of the sulphite alone. The effect extends beyond p_H 5·7 in either direction. When solutions of cysteine hydrochloride and $HgCl_2$ are mixed, a white precipitate of the mercaptide forms immediately. It is readily soluble in citrate buffer at p_H 5·7 and in strong acids. $HgCl_2$ does not affect the coloration developed by ferrous salt. 0·2 millimol. of $HgCl_2$ will reduce the coloration given by laevulic acid *plus* sulphite by about 20 %. $HgCl_2$ gives a white precipitate with uric acid at p_H 5·7. The precipitate is not soluble in citrate buffer but is readily dissolved by sulphite, by thiosulphate, by $CdCl_2$, by $ZnCl_2$ and by large quantities of NH_4Cl.

The heavy metals do not appear to influence the colorations given by laevulic acid or furfuraldehyde alone to a marked extent.

The coloration developed by sulphite alone may be increased considerably in the more acid mixtures by addition of much salt. Thus at p_H 5·7, 50 millimol. of Na_2SO_4 may nearly treble the coloration developed by the sulphite.

Ammonium chloride in quantity (5 millimol.) at p_H 5·7 increases the rate at which the coloration given by cystine *plus* sulphite develops, whereas large amounts of citrate buffer rather diminish the rate. NH_4Cl does not influence the coloration developed by ferrous salt but it may increase that given by cysteine alone by about 1 %. 5 millimol. of NH_4Cl increase the coloration given by 10 mg. of laevulic acid *plus* sulphite by about 18 % at the same p_H. It also greatly increases the coloration given by furfuraldehyde *plus* sulphite.

NH_4Cl does not appear to influence the colorations developed by laevulic acid or furfuraldehyde alone to a marked extent.

Reduction of the reagent by mixtures of substances.

The type example is the reaction by which cystine may be estimated. Cystine alone does not reduce the reagent. Sulphite reduces but slightly. A mixture of the two reduces strongly and the final coloration is virtually proportional to the cystine present provided this is not infinitesimal and that sufficient reagent is employed. Assuming that the sulphite contributes its full individual share to the total coloration developed in presence of cystine, one may calculate the departure to be expected from linearity of depth of coloration with cystine, assuming that only a small amount of sulphite is involved in converting the cystine to the same degree each time into something that will reduce the reagent. The calculated departure from linearity is several times in excess of that actually found. In other words the sulphite appears not to contribute its own coloration completely.

The same phenomenon is more easily appreciated from the curves in Fig. 1, showing the colorations developed by ferrous salt in absence and presence of sulphite. Here it is obvious that superimposing a sulphite curve on one given by ferrous salt alone will not lead to a curve of the type given by ferrous salt *plus* sulphite. As the displacement between the curves for ferrous salt with and without sulphite is small, and about the same at all p_H values, it is believed to be due to the sulphite precluding aerial oxidation of the ferrous salt rather than anything else.

Under certain conditions of volume, *etc.* and in the presence of 2 mg. of cystine, 1 millimol. of sulphite, and 2 cc. of reagent at p_H 5·7, 10 mg. of pyruvic acid, 10 mg. of laevulic acid, and 2 mg. of furfuraldehyde increased the coloration developed, by 0, 1 and 1·5 % respectively; whereas if the colorations had been additive the increases would have been 1, 8 and 11 % respectively.

Similarly the products of acid digestion of carbohydrates have been found to contribute only a fraction of the coloration they develop with sulphite to the total coloration obtained when fair quantities of cystine, ferrous salt, or cysteine are present.

Analysis and discussion of the experimental data.

The effects noticed when NH_4Cl and the heavy metal chlorides are added to laevulic acid or furfuraldehyde in presence of sulphite are really enhancement and inhibition of the rate of colour development. If the colour reactions are allowed to proceed for 5 hours instead of 5 minutes, the effects mentioned are almost lost.

The same might be said of the effects these substances exert in the case of cystine and cysteine. The actions of $CdCl_2$ as an inhibitor and of NH_4Cl as an enhancer are certainly upon the rate of colour development rather than upon the final equilibrium that might be attained, though this also might be affected. It is not unlikely that the action of $HgCl_2$, apparently protection of the —SH group by mercaptide formation, is of the same type, the effect being so pronounced however that no noticeable colour is developed even after some hours. The effects of these three heavy metal chlorides thus increase with their tendency to behave as non-polar substances and this is the order of the appearance of the metals in the same sub-group of the periodic system.

It is commonly stated that bisulphite reacts with only the lower members of ketonic series to form bisulphite compounds. The increased colour development noticed when furfuraldehyde and laevulic acid reduce the reagent in presence of sulphite cannot be explained. It can hardly be due to bisulphite compound formation since, for example, if a large excess of furfuraldehyde be used, extremely little colour is developed. This inhibiting effect, however, like that of acetone, is believed to be due to bisulphite compound formation.

As Wu points out in his examination of the complex acids, the "reducibility" of these substances by most reducers is small. He concludes that very few of the heavy metal atoms in the whole bulky complex are reduced as a rule, and that phospho-18-tungstic acid loses one atom of oxygen when reduced by ferrous salt in alkaline solution.

The colour reaction may be written:

$$\text{reagent} + \text{reducer} \rightleftharpoons \text{reduced reagent} + \text{oxidised reducer} \quad \ldots\ldots(1),$$

but this form involves the assumption that a true equilibrium may be reached. Until some small amount of the reagent is reduced the oxidation potential of the reagent system is virtually infinite.

In referring to oxidation-reduction potentials of the reagent and reducer systems we really imply that the potential of the one system is linearly related to $ln \frac{[\text{reagent}]}{[\text{reduced reagent}]}$ and that of the other to $ln \frac{[\text{reducer}]}{[\text{oxidised reducer}]}$. These relationships might not apply, but assuming that they do it is not difficult to see why different reducers might give different amounts of colour and why weak reducers fail to develop their full colorations in presence of strong ones. When cysteine, ferrous salt, cystine *plus* sulphite or Na_2S (the last in alkaline solution) is the reducer, the balance represented by the arrows is well in favour of the right-hand side of the equation. With other substances the balance

seems to be in favour of the left-hand side. This is evidenced by the greater colour developed when more reagent is used, as in the case of uric acid; the lack of proportionality between colour and amount of reducer, the poor reduction effected by large amounts of potential reducer, *etc.* The actual magnitudes as well as the ratios of the forward and backward velocity constants must be important factors in some instances.

Cysteine and the other strong reducers certainly seem to achieve some sort of balance. At p_H 5·7 with 2 cc. of reagent, 1, 2 and 4 mg. of cystine *plus* sulphite or of cysteine alone, or 1 and 2 mg. of cysteine *plus* sulphite, give amounts of colour almost strictly proportional to the amounts of cystine or cysteine present. With 6 mg. of cystine *plus* sulphite or 6 mg. of cysteine or 3 mg. of cysteine *plus* sulphite, the departure from linearity is slight, but with say 10 mg. of cysteine or a corresponding amount of ferrous salt for example the departure is considerable (20 %). With 80 mg. of cysteine the departure is about 800 %. The relation between colour and amount of cysteine follows approximately the same trend at p_H 8·4, though the balance would appear to be rather more in favour of the right-hand side of the equation than it is at p_H 5·7.

The reagent is probably almost exhausted when such large amounts of cysteine are employed. Assuming that all the tungsten present in 2 cc. of reagent exists in the complex and that each molecule of the complex may lose only 1 atom of oxygen to cysteine or ferrous salt, then if 80 mg. of cysteine could reduce all the reagent the departure from linearity would be 670 % instead of 800 %. If Wu's findings are correct then at least 80 % of the tungsten present in the reagent employed here exists in the complex. But the balance cannot be so completely in favour of the right-hand side of the equation (*cf.* the departure with 10 mg. of cysteine) and hence it is possible that nearly 100 % of the tungsten exists in the complex.

It will be seen later that cysteine is oxidised to cystine by the reagent. Whatever the nature of the balance achieved by cysteine may be, it cannot at present be interpreted in known physico-chemical terms. The potential of a cysteine-cystine system at an inert electrode cannot be described by Peter's equation. In the equation that describes the potential,

$$E = E_0 - \frac{RT}{F} ln\,[\text{cysteine}] + \frac{RT}{F} ln\,[H^+],$$

the concentration of cystine finds no place at all, and attempts to explain it in terms of Wieland's theory of hydrogen acceptors have recently been discredited by Michaelis and Flexner [1928].

Those reducers which seem to establish an early balance with the reagent practically all give increasing colorations at p_H values increasing up to about 7·5. Above p_H 6·5 the predominating sulphite ion in a sulphite solution will be the $SO_3^=$ ion. With the exception of such substances as uric acid the colorations thereafter decline. This effect is offset by that observed with the stronger reducers as p_H increases above about 7·5. That the colorations with ferrous

salt and the other strong reducers should increase, strongly suggests that the reagent is subject to a different reduction from that occurring in the acid solutions or that the reagent is of different form in the two zones. The former possibility seems to be ruled out by the discussion on the extent to which 2 cc. of the reagent are reduced by 80 mg. of cysteine at p_H 5·7 and p_H 8·4. The latter possibility, if accepted, revives interest in Wu's claim that the 1–18-acid may exist in two forms A and B. The A form which is said to give nearly 30 % more colour than the B on total reduction is also said to be more easily reduced than the B form. With this in mind, the curves in Fig. 1 might suggest that the two forms are in equilibrium at any p_H, the B form predominating at low p_H and the A at high, though one would expect the weak reducers to give more instead of less colour at high p_H if this were so, and that a new equilibrium is only slowly attained when the active mass of one form is altered. This view is supported by the observations made on the effects of the order of adding the materials in alkaline solution.

The very rapid fading encountered with quinol as reducer cannot be explained. Excess bromine readily destroys the blue colour developed by the substances described in this paper, but H_2O_2 is usually almost without effect. Saturation with acetone sometimes causes complete bleaching in a few seconds.

The slow fading observed with cysteine, cystine *plus* sulphite, *etc.*, as reducers seems to be associated in some way with decomposition of the 1–18-acid. Its rate increases with the p_H and to some extent with salt concentration and type. Thus large amounts of $ZnCl_2$ or $CdCl_2$ very considerably increase the rate. The stock reagent used in all this work is buffered at about p_H 2·1 and remains stable for years. If some reagent is brought to p_H 8·4 and buffered it will be found to lose practically all its reducibility after some hours. Buffered at p_H 5·7 the reagent will "work" for a few days. Thereafter it loses its pale apple-green colour, which becomes pale sky-blue, and is not reduced by cysteine or cystine *plus* sulphite. Ferrous salt reduces it slightly giving a blackish-green tint. Nothing is precipitated from the reagent kept at p_H 5·7 even after standing for 5 months at room temperature. This form of decomposition of the reagent is not at present understood.

In reference to the colorations given by ferrous salt, cysteine and the other members of that group it has been concluded that the reducers are virtually fully oxidised to other substances by a relatively small excess of reagent, and from the colorations developed by ferrous salt during its oxidation to the ferric state one may associate a certain amount of colour with a certain amount of reduction.

In extreme alkaline regions and with plenty of reagent, the coloration given by Na_2S corresponds with the oxidation of both atoms of hydrogen in the H_2S molecule. Unless sulphite is present the solution is clouded by free sulphur and as the sulphite does not alter the colour, as far as one may judge between cloudy and clear solutions, it is believed that it merely combines with the free sulphur to form thiosulphate. At lower p_H values nothing like

full colour development is obtained in 5 minutes but it is approached with longer periods of standing. The solutions always smell of H_2S during colour development and unless plenty of reagent is used to diminish the time necessary for almost complete oxidation appreciable amounts of H_2S may be lost. At still lower p_H values the H_2S and sulphite must react in some measure to form thionic acids.

The cysteine hydrochloride used was not perfectly dry and in consequence the amounts weighed out for standard solutions were probably deficient in cysteine by 1 or 2 %. It had been prepared by reducing cystine with tin and hydrochloric acid and strict precautions were taken to prevent its re-oxidation in the course of separation. It certainly contained only the minutest trace of cystine, if any.

Now if the reagent, in being reduced by cysteine, oxidises the cysteine to cysteic acid, then, focusing attention purely upon the amounts of colour given by cysteine and by cystine *plus* sulphite, one may say that the facts do not disagree with the view that the sulphite reduces the cystine to cysteine. But if this were so, why should cysteine *plus* sulphite develop just twice the colour given by cysteine alone? The hypothesis that the sulphite reduces the cystine to cysteine and that this is oxidised by the reagent to cysteic acid, is rendered untenable by that fact alone. The second part of the hypothesis is independently invalidated by a consideration of the amounts of colour given by cysteine alone and by ferrous salt. The colour given by cysteine alone corresponds to within a few units % with the oxidation of the cysteine to cystine, not to cysteic acid. And according to this fact just established, the first part of the hypothesis must be incorrect, for if not, the colours developed by cysteine *plus* sulphite and cystine *plus* sulphite should continually increase in intensity until the sulphite is exhausted. The rôle played by cystine would then be that of catalyst to the reduction of the reagent by the sulphite, and this obviously is not the case.

The anomaly is bound up with the belief that sulphite reduces the cystine to cysteine under the conditions obtaining in the estimations. It seems that this is quite incorrect and must be abandoned. Other evidence which might appear to be against it, but is not necessarily so, is the following. Sulphite reduces the reagent only slightly, yet cysteine during its oxidation to cystine reduces strongly. Is it likely therefore that the sulphite could reduce cystine appreciably under the same conditions? The extent of reduction unfortunately cannot be calculated because the sulphite-sulphate system is irreversible under these conditions and the cysteine-cystine system is anomalous.

The following table (p. 2157) summarises the position with respect to colour development by ferrous salt, cysteine, cysteine *plus* sulphite, cystine, cystine *plus* sulphite, Na_2S, and Na_2S *plus* sulphite, the last two only in alkaline solution. The unit of colour is to be associated with 1 atom of reducing hydrogen. RSH and R—S—S—R represent cysteine and cystine respectively.

When cysteine is first carefully oxidised to cystine at p_H 5–6 by chromate,

or by H_2O_2 *plus* a trace of ferrous salt, it gives on subsequent addition of sulphite only half the colour it would have given if it had not first been oxidised.

	Amount and nature of reducer	Units of colour developed
I	$2r$ Fe^{++}	$2r$
II	$2r$ RSH	$2r$
III	$2r$ RSH + sulphite	$4r$
IV	r R—S—S—R	0
V	r R—S—S—R + sulphite	$2r$
VI	$2r$ H_2S (alkaline)	$4r$ cloudy
VII	$2r$ H_2S + sulphite (alkaline)	$4r$

The equation

$$R—S—S—R + H_2SO_3 \text{ aq.} \longrightarrow 2RSH + H_2SO_4 \text{ aq.} \quad \ldots\ldots(2),$$

is, as we have seen, quite unable to account for the facts.

Let it now be supposed that the action of sulphite on r molecules of cystine is to produce r molecules of cysteine *plus* s molecules of a substance X which is unable to reduce the reagent. The following formulation will suffice:

$$r\,R—S—S—R + u SO_2 + v H_2O \rightleftharpoons r\,RSH + sX \quad \ldots\ldots(3)^1.$$

It has also been established that:

$$2r\,RSH + \text{reagent} \rightleftharpoons r\,R—S—S—R + \text{reduced reagent} \ldots\ldots(4),$$

the balance being found almost completely in favour of the right-hand side of the equation. The fact that the colour given by cysteine is doubled in presence of sulphite now admits of easy explanation.

Starting with equation (4) and with case II in the table, $2r$ of cysteine first give $2r$ units of colour and are converted into r of cystine. If sulphite is present then r of cystine according to equation (3) yield another r of cysteine which react with the reagent to give another r units of colour and $\frac{1}{2}r$ of cystine, and so on. The total colour developed is therefore

$$2r + r + \tfrac{1}{2}r + \tfrac{1}{4}r + \tfrac{1}{8}r + \ldots\ldots$$

which, in the limit, becomes $2r + 2r$ or $4r$.

The colour developed by r of cystine alone is 0, and by r of cystine *plus* sulphite is $2r$. The case admits of similar explanation to that given above. The initial $2r$ of colour is of course missing and the total colour developed is merely that of the summation, $r + \frac{r}{2} + \frac{r}{4} + \textit{etc.}$, becoming $2r$ in the limit.

Thus equations (3) and (4) account satisfactorily for the known facts. The reaction expressed by (4) is quite rapid over the p_H range 5–8·4, the greatest rate of colour development occurring in the most alkaline solutions. From the very low rate of colour development with cystine *plus* sulphite at low p_H values compared with the rate at higher values, it has been concluded that (*a*) the reaction velocities in equation (3) are very small in the acid region,

[1] The double reaction arrows in this and following equations must be taken only to represent a possible equilibrium of some sort, not necessarily of the type such equations ordinarily represent.

or (*b*) the velocities may be high but with equilibrium well in favour of the left-hand side in acid solutions, or (*c*) both (*a*) and (*b*) might apply.

From the point of view of interest in the *X* compound of equation (3), the following considerations and experiments are appended.

The simplest form equation (3) may take is

$$R—S—S—R + SO_2 + H_2O \rightleftharpoons RSH + RHS_2O_3 \quad \text{.....(5),}$$

in which sX becomes RHS_2O_3.

Equation (5) represents a reaction somewhat similar to that given for the action of cyanides upon cystine

$$R—S—S—R + NaCN \rightleftharpoons RSNa + RCNS \quad \text{.....(6).}$$

An alternative to equation (5) as a particular case of equation (3) would be

$$3R—S—S—R + 8SO_2 + 5H_2O \rightleftharpoons 3RSH + 2H_2S_4O_6 + 3RSO_3H \quad \text{...(7)}$$

in which the *X* compound would occur as a mixture of tetrathionic and cysteic acids, both of which are fairly stable and not reactive toward the reagent. On the evidence thus far submitted equations (5) and (7) would perhaps have equal claims to being the correct one.

Organic thiosulphates of the form postulated in equation (5) are by no means unknown. Purgotti [1892], investigating the thiosulphonyl derivative of glycollic acid, prepared it by treating chloroacetic acid with sodium thiosulphate. Aqueous solutions of the salts are not readily decomposed by boiling, but, in contrast to the action of mineral acids upon inorganic thiosulphates, boiling hydrochloric acid converts the barium salt of the thiosulphonyl derivative of glycollic acid, for example, into barium sulphate and thioglycollic acid.

It was found that a stream of SO_2 could be blown into a solution of cystine in excess of hydrochloric acid without any chemical change occurring. No sulphate was produced and none of the cystine was affected in any way. On boiling to expel SO_2 the solution was found to contain the original cystine and hydrochloric acid and nothing else. The original cystine could be accounted for colorimetrically and the total sulphur content corresponded with the amount of cystine present.

A mixture containing cystine and $NaHSO_3$ was made alkaline at p_H 12–13, then acid, alkaline again, and acid again, all in the cold. No sulphate was produced. An aliquot was allowed to develop its colour with the reagent in presence of more sulphite and the colour was compared with that of a standard cystine + sulphite solution containing the same amount of cystine as that originally present in the aliquot. There was a 7 % loss of colour. Another aliquot was boiled to expel SO_2 and then allowed to reduce the reagent in presence of sulphite. There was a gain in colour developed of 77 %. In Part II of this series, methods for estimating cysteine and cystine will be discussed in detail. Applying them to analysis of the boiled aliquot it was found that

81 % of the cystine had been converted into cysteine, 13 % remained unchanged, and the balance, 6 %, had been lost to colorimetric estimation. Gravimetric analyses of sulphate and total sulphur confirmed these results and revealed that the sulphur of the 6 % cystine lost to colorimetry remained in the solution, probably as cysteic acid.

These experiments throw a little light on the nature of the *X* compound which is certainly elusive but not necessarily chimerical. If the thiosulphonyl derivative of α-aminopropionic acid is the *X* compound and reacts with boiling mineral acid as does Purgotti's thiosulphonyl derivative of glycollic acid, the observations are automatically explained. There can really be little doubt that equation (5) is the correct form of equation (3).

Summary.

1. The reduction of solutions of phospho-18-tungstic acid by different types of substances has been investigated colorimetrically under varying conditions, especially in respect to varying p_H. Some attempt has been made to describe the results in general terms of reaction velocities and equilibria. Reductions by cysteine have been examined in some detail.

2. Evidence has been adduced to show that the reaction between cystine and sulphites in cold aqueous solution is not a "reduction" in the ordinary sense of the term, but that it is most probably expressed by the equation, $R—S—S—R + H_2SO_3 \rightleftharpoons RSH + RHS_2O_3$, where R—S—S—R and RSH represent cystine and cysteine respectively.

I wish to express my indebtedness to Sir Charles J. Martin for his interest in this work and for correcting the manuscript and arranging for its publication.

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CCLIV. THE APPLICATION OF PHOSPHO-18-TUNGSTIC ACID (FOLIN'S REAGENT) TO THE COLORIMETRIC DETERMINATION OF CYSTEINE, CYSTINE AND RELATED SUBSTANCES.

II. THE DETERMINATION OF SULPHYDRYL COMPOUNDS AND DISULPHIDES ALREADY EXISTING IN SOLUTION.

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The principles underlying the new methods will be indicated briefly, but to form an idea of the magnitudes and likelihood of errors and for a proper understanding of the principles involved, it will be necessary to refer to Part I of the series [1932].

By estimating cysteine and cystine in acid solution all the disadvantages of alkaline colour development may be avoided. The disadvantages of acid colour development include slight loss in colour and diminution in rate of development. For all round utility a p_H of 5·7 has been selected, and to minimise colour development by extraneous reducers and for the sake of economy, only small amounts of reagent are employed. A cystine *plus* sulphite standard is used for comparison.

The cysteine is allowed to develop its coloration in absence of sulphite and its estimation in absence of extraneous reducers is quite simple. In coloration developed, 1 mol. of cystine = 2 mol. of cysteine, or 2 mg. of cystine = 2·017 mg. of cysteine. When extraneous reducers are present their effects must be found by artifice. In the presence of mercuric chloride cysteine gives no coloration at all, and ferrous salt is used to find the contribution of the extraneous reducers to the total coloration developed. Since $HgCl_2$ will form a precipitate with uric acid, and because it influences slightly the coloration developed by some extraneous reducers, it is necessary to have a fairly large amount of zinc chloride present to prevent precipitation and to provide an initial "heavy metal" effect upon the weaker reducers so that the effect of the $HgCl_2$ will be diminished. To nullify the inhibiting effect of the $ZnCl_2$ upon the rate of colour

development by cysteine, it is desirable that some NH_4Cl be present also. The $ZnCl_2$ and NH_4Cl are incorporated with the citrate buffer used in preparing standard and unknowns.

Cystine is estimated with sulphite present and the estimation is simple when extraneous reducers are absent. The same buffer as is used in estimating cysteine is employed. When extraneous reducers are present, mercuric chloride and ferrous salt are used as in the corresponding cysteine estimation.

Provided extraneous reducers are absent, the estimations of cysteine and cystine together are not difficult. In absence of sulphite the coloration is due to cysteine alone. The cystine develops its usual colour in presence of sulphite and the cysteine develops twice as deep a coloration and hence, subtracting twice the value obtained in absence of sulphite from that obtained in presence of sulphite, the nett amount represents the cystine. In so far as the cysteine in the unknown solution may be converted fairly rapidly into cystine by aerial oxidation, it might be preferable to record the "total cystine" (cysteine *plus* cystine) content by subtracting the value of the coloration obtained in absence of sulphite from that obtained in its presence and calculating the nett amount on the basis of 2 mg. of cystine = 2 mg. of "total cystine." The solution which develops its coloration in presence of sulphite should develop most of its cysteine coloration in absence of the sulphite, which is added after a short interval. By this precautionary measure the "total cystine" might be estimated quite accurately even if a considerable amount of the cysteine were to suffer aerial oxidation during colour development. Provision is made for this in the method, but tests so far have shown that the precaution is not necessary.

When extraneous reducers are present they may contribute different amounts of colour in absence and presence of sulphite. Mercuric chloride and ferrous salt are used in absence and presence of sulphite to find the two contributions.

The use of mercuric chloride renders these methods highly specific for sulphydryl compounds and disulphides, and in analysing any solution it is wise to assume that extraneous reducers are present and to proceed accordingly.

Neither cysteine nor cystine is extracted from acid aqueous solution (p_H less than 3) by ether, but part of the cysteine may be oxidised to cystine in the process of extracting a solution. On the other hand laevulic acid, pyruvic acid, furfuraldehyde, and similar substances may all be extracted though never completely. Their contributions to the coloration developed may thus be greatly reduced and the possible error in a cystine estimation thereby diminished. Four extractions with twice the volume of ether each time will remove approximately four-fifths of the laevulic and pyruvic acids and practically all the furfuraldehyde. Ether extractions introduce a decided degree of specificity for cysteine and cystine among the sulphydryl compounds and disulphides. The condition that these compounds should remain in the aqueous acid solution is probably that they contain a strongly basic group like $—NH_2$.

Complications arise when the unknown solution is coloured initially. Selective absorbents should never be used as their selectivity can never be perfect and is frequently very poor. Theoretically the compensating colorimeter is the instrument to be employed and the blank used with the standard must or must not contain sulphite according to its presence or absence in the unknown. There is always some uncertainty in the matter of the blanks because one cannot know what effect the reagent would have upon the adventitious coloration.

If the adventitious coloration is the same as that being developed its value may be subtracted from the readings obtained when an ordinary colorimeter is employed. Even when the adventitious coloration is at the other end of the spectrum, as in protein hydrolysates, an experienced operator may blind himself to it, if it is not extremely pronounced, and achieve excellent results with an ordinary colorimeter. It is the author's experience that most compensating instruments fail to survive even those simple tests to which an ordinary instrument is put before being used.

The reagents, which are listed below, should be filtered through sintered glass if necessary. The list is followed by directions for making estimations.

Phospho-18-tungstic acid reagent. This is prepared according to the directions of Folin and Marenzi [1929] with separation of any molybdenum present according to their plan. It may be looked upon as containing per litre 100 g. of the salt $Na_2WO_4, 2H_2O$, 172 g. of H_3PO_4, and 16·1 g. of LiOH. The sodium tungstate should be prepared by dissolving the necessary amount of precipitated WO_3 in the necessary amount of NaOH solution. If the reagent fails to comply with the following requirements it must be discarded. (1) It must give no coloration with tyrosine or tryptophan at any p_H. (2) At p_H 5·7 and 8·4, 4 mg. of cystine in presence of 2 cc. of reagent and 1 millimol. of sulphite must give not less than 3·95 times the coloration developed by 1 mg. of cystine. (3) At p_H 5·7 and 8·4 and in absence of zinc and ammonium salts, 80 mg. of cysteine with 2 cc. of reagent must give almost exactly seven times the coloration developed by 2 mg. of cysteine.

Sodium acetate solution: a 4*M* solution containing 544 g. of the salt $C_2H_3O_2Na, 3H_2O$ per litre.

Composite citrate buffer. This contains 105 g. of crystalline citric acid $C_6H_8O_7, H_2O$, 52·5 g. of NaOH, 13·6 g. of $ZnCl_2$ and 26·8 g. of NH_4Cl per litre. It is best prepared by shaking the citric acid and NaOH with about 500 cc. of water with constant cooling to avoid boiling, until entirely dissolved. The $ZnCl_2$ is dissolved in 20 cc. of water and added to the cold solution, and this is followed by the NH_4Cl dissolved in about 200 cc. of water. The mixture is diluted to 1 litre.

Buffered sulphite. 9·5 g. of sodium metabisulphite ($Na_2S_2O_5$) are dissolved in about 70 cc. of water, 15 cc. of 4*M* sodium acetate solution are added and the mixture is diluted to 100 cc. This *M* sulphite solution will keep for months in a stoppered bottle.

Mercuric chloride: a 0·1 *M* solution containing 2·72 g. of $HgCl_2$ per 100 cc. of solution.

Ferrous salt: a *M*/12 Fe^{++} solution containing 3·268 g. of Mohr's salt ($FeSO_4$, $(NH_4)_2SO_4$, $6H_2O$) and 8·5 cc. of *N* H_2SO_4 per 100 cc. It will keep for months in a well-stoppered bottle and may be titrated against potassium permanganate to check alteration. The solution is diluted 1 in 8, 1 in 9, 1 in 10, *etc.*, before use in the estimations.

Standard cystine solution. 0·5 g. of pure cystine (*l*-cystine is most easily obtained) is dissolved in 50 cc. of *N* hydrochloric acid, and diluted to 500 cc. to provide a *M*/240 solution in 0·1 *N* HCl, containing 1 mg. of cystine per cc. The solution will keep indefinitely.

Estimations in general.

All estimations are carried out in standard 100 cc. flasks. Standard and unknown should be of approximately the same colour intensity (70–150 %) and must be prepared together. In reference to the 5–10 minute interval allowed for colour development, one of about 7 minutes is to be preferred. The solutions should be read within 30 minutes of being diluted to the mark.

The standard cystine solution is acid and every 1 cc. requires 0·25 cc. of the sodium acetate solution to be brought to p_H 5·7. Aliquots of the unknown cysteine, cystine, and mixed solutions must be titrated to p_H 5·7 with the sodium acetate solution, using bromocresol purple indicator. The appropriate amounts of acetate must be included with the larger amounts mentioned in the schedules below when preparing the standards and unknowns.

Estimation of cysteine.

Unknown. 10 cc. of citrate buffer, 2 cc. of reagent and 2 cc. of sodium acetate solution are mixed in a flask in that order, and to the mixture is added the chosen aliquot of unknown cysteine solution.

Standard. To a mixture of the same composition as that used for the unknown is added the necessary amount of cystine solution and this is followed by 1 cc. of buffered sulphite.

After 5–10 minutes the solutions are diluted and compared. The apparent cysteine content will be $x + a$ mg., where x is the true amount; in absence of extraneous reducers a will be zero. If extraneous reducers are present a is found as follows. Another unknown is prepared just as before, but, prior to adding the unknown cysteine solution, 1 cc. of the mercuric chloride solution is added to the mixture. The amount of coloration developed, calculated as cysteine, will correspond with A mg. By trial and error in a series of solutions, the amount of ferrous salt necessary to make the total coloration agree closely with the original apparent $x + a$ mg. of cysteine, is found. A new unknown is prepared containing the mercuric chloride and unknown cysteine solutions followed by the necessary amount of ferrous salt, and compared with a new

standard containing precisely the same amount of ferrous salt but none of the unknown cysteine solution. The contribution of the extraneous reducers to the colour developed by the ferrous salt is found to be a_1 in terms of mg. of cysteine. a_1 may be different from A but it is nearly the same as a, and hence x is found from $x + a - a_1$.

Estimation of cystine.

The standard and unknown are prepared in precisely the same manner as the standard cystine *plus* sulphite solution used in estimating cysteine. The apparent amount of cystine found will be $y + b$ mg., where y is the true amount, and b is zero when extraneous reducers are absent. If extraneous reducers are present then b is found as follows. Another unknown is prepared just as before and containing sulphite, but prior to adding the unknown cystine solution 1 cc. of the mercuric chloride solution is introduced. The coloration will correspond with b mg. of cystine. By trial and error the amount of ferrous salt necessary to make the total coloration agree closely with the original apparent $y + b$ mg. of cystine is found. A new unknown is prepared containing mercuric chloride, the unknown cystine solution, and sulphite, and after colour development has proceeded for about 1 minute the necessary amount of ferrous salt is added. Colour development is allowed to continue for the remainder of the 5–10 minutes and on dilution the solution is compared with a new standard containing mercuric chloride, sulphite, and the same amount of ferrous salt, but none of the unknown cystine solution. The contribution of the extraneous reducers to the colour developed by the ferrous salt in presence of sulphite is thus found to correspond with b_1 mg. of cystine. Identifying b_1 with b, y is found from $y + b - b_1$.

Estimation of cysteine and cystine when present together.

The procedure is merely a combination of those used in estimating cysteine and cystine. Suppose there are x mg. of cysteine and y mg. of cystine present in a chosen aliquot. In absence of extraneous reducers the colour developed without sulphite will correspond with the x mg. of cysteine. With sulphite the colour will correspond with $2x$ mg. of cysteine *plus* y mg. of cystine. Hence both x and y are found. If extraneous reducers are present the coloration in absence of sulphite will correspond with $x + a$ mg. of cysteine, and in presence of sulphite it will correspond with $2x$ mg. of cysteine *plus* $y + b$ mg. of cystine. In absence of sulphite mercuric chloride is used first to find a and later with ferrous salt to find a_1. In presence of sulphite it is used first to find b and then with ferrous salt to find b_1. a_1 is assumed identical with a, and b_1 with b, and hence both x and y may be found.

None of the coloured solutions used in estimating cysteine, cystine, and mixtures of the two, should exceed in intensity the coloration developed by 4 mg. of cystine in presence of sulphite.

SUMMARY.

Colorimetric methods involving the use of phospho-18-tungstic acid (Folin's reagent) are described for the estimation of sulphydryl compounds and disulphides, with especial reference to cysteine and cystine.

The estimations may be performed in the presence of large amounts of extraneous reducers. Under such conditions the methods are rather tedious but have been found to yield reasonably accurate results.

The author wishes to express his indebtedness to Sir Charles J. Martin for continued interest and assistance in publication.

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CCLV. THE RECIPROCAL RELATIONSHIP OF CALCIUM AND INORGANIC PHOSPHORUS OF THE BLOOD OF SHEEP.

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(*Received May 14th, 1932.*)

Howland and Kramer [1923] showed that in rickets the concentration product of calcium and inorganic phosphorus falls below a certain critical level. Their work suggested a fundamental relationship between the levels of Ca and inorganic P of the blood.

Bourne and Campbell [1932] by simultaneous determinations of calcium and inorganic P of the blood in rabbits have shown that the concentrations of these two substances bear a reciprocal relation to each other. Their work is fully supported by the work of Dupré and Semeonoff [1931]. On the other hand Theiler, Green and du Toit [1927] found in their extensive work on cattle that the blood-Ca remained normal even with an inorganic P of one quarter the normal figure. This contradiction might suggest that in this respect ruminants differed fundamentally from rabbits.

Recent work by Auchinachie and Fraser [1932], however, on the blood of sheep fed a Ca-deficient ration has shown that an abnormally low serum-Ca may be accompanied by an abnormally high inorganic P, and that there is evidence of a reciprocal relationship between the Ca and inorganic P of the blood of sheep.

Methods.

In November 1930 five sheep were put on a Ca-deficient diet. Five other sheep were put on the same diet *plus* 5 cc. of cod-liver oil per sheep per day. Simultaneous estimations of blood-Ca and inorganic P were made at intervals in each individual of both groups. Both groups were kept out of doors.

Results.

The average figures for serum-Ca are given in Table I. In Group I the serum-Ca fell to the low level of 7·06 mg./100 cc. in March rising to 8·15 in June. During the same period (November–June) the addition of cod-liver oil maintained the serum-Ca at a normal level.

Table I. *Ca in mg./100 cc.*

	January	March	June
Group I (Ca-deficient)	10·65	7·06	8·15
Group II (Ca-deficient + cod-liver oil)	11·10	10·25	10·00

The average figures for inorganic P are given in Table II. It is apparent that while the P in both groups shows a seasonal increase, the blood-P in the Ca-deficient group was very much higher than when cod-liver oil was fed, particularly in March.

Table II. *P in mg./100 cc.*

	January	March	June
Group I	5·63	10·53	9·15
Group II	6·11	7·29	7·66

It is evident that there is a significant difference between the two groups both in blood-Ca and blood-P.

There is, however, a remarkably close correspondence between the Ca and P concentration products of the two groups, as is shown by Table III.

Table III. *Ca × P concentration product.*

	January	March	June
Group I	67·1	72·3	77·3
Group II	67·8	74·7	76·3

In each group there is evidence of a seasonal rise in the product, but at any one period there is a close correspondence between the products of both groups. This is important in view of the divergent values obtained for Ca alone and for P alone. It suggests that the Ca and P levels of the blood in sheep are reciprocally related, the P level rising proportionately to the fall in Ca and *vice versa*, a result in keeping with the results of Bourne and Campbell and of Dupré and Semeonoff on rabbits.

Discussion.

These results on sheep were obtained on small groups and have not yet been repeated. They must therefore be regarded as of a preliminary nature. At the same time the evidence of a reciprocal relationship in this particular case is perfectly clear and is in agreement with similar results on rabbits. In discussing the possible interpretation of this relationship, there are several main possibilities to consider. In the first place the basal diet whilst deficient in Ca was adequate in P. It is conceivable that the blood-P might rise owing to the Ca-deficiency leading to a reduced rate of formation of insoluble compounds of Ca and P in the intestine. This interpretation is ruled out by the effect of cod-liver oil, where no extra lime entered the intestines, and yet the blood-P fell markedly below that of the Ca-deficient group.

It seems more probable that the phenomena of reciprocal relationship of

the Ca and P of the blood are bound up with the metabolism of bone in the manner suggested in the following hypothesis.

As a result of deficiency in the food, the amount of Ca absorbed from the gastro-intestinal tract is insufficient to maintain the normal level of serum-Ca, and the calcium reserves of the bones are utilised to supply the needs of more essential tissues. But since Ca is present in bone mainly as calcium phosphate the transference of calcium from bone to blood will be accompanied by a simultaneous transference of phosphorus. This view is supported by the work of Evans [1930] on swine, and of Bekker and Rousseau [1930] on sheep, which shows that neither a Ca nor a P deficiency in the diet alters the percentage composition of the bones. Provided that P is being assimilated in normal amount, the liberation of bone phosphate will lead to a rise in the blood-P level, and the extent of this rise will be proportionate to the rapidity of mobilisation of the calcium reserves in the bones. Cod-liver oil by raising the serum-Ca level, reduces the demand on the Ca bone reserves, decreases the liberation of bone phosphate, and hence lowers the blood-P level. On this hypothesis the blood-P will rise in response to a fall in serum-Ca, and fall in response to a serum-Ca rise, thus leading to a reciprocal relationship of Ca and P in the blood, and to a relative constancy of the Ca × P concentration product.

In cases where both calcium and phosphorus are deficient in the diet, or where conditions favouring assimilation are sub-normal, it is obvious that this reciprocal relationship will not be clearly shown.

Summary.

1. In sheep under outdoor conditions, fed a basal ration deficient in calcium, or such a ration supplemented with cod-liver oil, the Ca × P product of Howland and Kramer tends to approach a constant, in spite of wide variations in serum-calcium and blood-inorganic phosphorus produced by differences in diet.

2. The relative constancy of the Ca × P product is shown to depend upon the reciprocal relationship of the calcium and phosphorus of the blood, the level of phosphorus rising as that of calcium falls, and *vice versa*.

3. It is suggested that this reciprocal relationship is due to factors involved in utilisation of the mineral reserves of the bones.

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CCLVI. THE RELATION OF THIOL COMPOUNDS TO GLUCOSE FERMENTATION.

By JUDA HIRSCH QUASTEL
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(*Received November 1st, 1932.*)

In the course of experiments on the toxicity of iodoacetic acid to biological processes, it was found that addition to the acid of sodium thiosulphate or thiol compounds brought about a considerable diminution in the intensity of the toxic effect. This observation led the writers to consider the possibility that thiol compounds may normally play an important part in the processes affected by iodoacetic acid. Experiments were accordingly devised to test this possibility, especially with regard to yeast fermentation. The results which are recorded below showed that both cysteine and glutathione markedly affect the relationship of aerobic fermentation of glucose by baker's yeast to the respiration in presence of glucose. It would seem that glutathione plays some controlling part in determining the relationship between aerobic fermentation and respiration of the cell in presence of glucose.

A. Experiments with iodoacetic acid.

Experiments with animals showed that the toxicity of iodoacetic acid was greatly diminished by admixture with sodium thiosulphate. For instance, an intramuscular injection of 3 cc. of a 2 % solution of iodoacetic acid (neutralised with NaOH) was invariably fatal to guinea-pigs, death occurring within 1 hour after the injection. When, however, 0·3 g. sodium thiosulphate was dissolved in this dose of iodoacetic acid death of the animal did not occur. A separate injection of thiosulphate did not relieve the animal from the toxic action of iodoacetic acid and it was apparent that the diminution of toxicity of this acid by the presence of thiosulphate was due to interaction between the two substances.

This was confirmed by iodimetric titration of sodium thiosulphate solution after the addition of iodoacetic acid. Two solutions were prepared (*a*) 50 cc. $N/100$ sodium thiosulphate, (*b*) 50 cc. $N/100$ sodium thiosulphate to which was added sodium iodoacetate to give a concentration of $N/250$. The solutions were placed at 37° for 3 hours after which titrations on 5 cc. with $N/100$ iodine solution were made. The titrations were (*a*) 5·0 cc., (*b*) 3·15 cc. The theoretical

figure for (*b*) was 3·0 cc. had there been complete interaction between one molecule of sodium thiosulphate and one molecule of iodoacetic acid.

Experiments were then carried out to determine whether the halogen atom of iodoacetic acid was sufficiently labile to react with the thiol groups of cysteine and glutathione.

In performing these experiments, advantage was taken of the fact that exposure of brain tissue to dilute iodoacetic acid resulted in an inability of the tissue to oxidise glucose and other sugars [Krebs, 1931; Quastel and Wheatley, 1932].

Fresh brain tissue was added to solutions containing (*a*) iodoacetic acid, (*b*) iodoacetic acid + cysteine, (*c*) iodoacetic acid + glutathione, (*d*) iodoacetic acid + sodium thiosulphate. It was found that the inhibitory action of iodoacetic acid on the oxidation of glucose by brain was much reduced by its previous admixture with thiol compounds or sodium thiosulphate.

The experimental technique, which involved the use of the Barcroft differential manometer, was that fully described in an earlier communication [Quastel and Wheatley, 1932]. Briefly it consisted in placing in the right-hand vessel of the manometer 1 cc. *M*/5 phosphate buffer solution p_H 7·4, 2 cc. saline (containing the iodoacetic acid or mixture of iodoacetic acid and sulphur compound) and 0·5 g. fresh chopped guinea-pig brain tissue. 3 cc. saline were placed in the left-hand vessel. The apparatus was shaken at the temperature of the bath (37°) for 2½ hours when the rate of autoxidation of the brain had fallen very considerably. Glucose was then added to the tissue to give a final concentration of 0·025 % and the new rate of oxygen uptake was observed. This was compared with the rate given by brain tissue which had not been treated with iodoacetic acid. The p_H of all solutions added to the brain tissue was 7·4—acids being neutralised with sodium hydroxide. The results are shown in Table I.

Table I.

Oxygen uptakes in mm.³ by 0·5 g. guinea-pig brain tissue (in presence of 0·025 % glucose) after treatment with iodoacetic acid and mixtures of iodoacetic acid and sulphur compounds. Autoxidation had been allowed to proceed for 2½ hours before addition of the glucose.

Mode of treatment	Time (in mins.) 30	60	90	120
Control (no iodoacetic acid)	144	312	474	618
M/4000 iodoacetic acid	52	95	124	150
,, + *M*/160 cysteine	108	235	356	482
,, + *M*/100 glutathione	128	270	408	548
,, + 0·7 % sodium thiosulphate	92	187	285	382
Cysteine *M*/160	124	266	404	538
Glutathione *M*/100	142	302	460	612
Sodium thiosulphate (0·7 %)	147	313	471	610

It will be observed that cysteine, glutathione and sodium thiosulphate diminish the toxic action of iodoacetic acid, a considerably greater rate of oxygen uptake being secured. Control experiments showed that none of those

sulphur compounds, when added alone, appreciably affected the oxygen uptake of brain tissue in the presence of glucose. Cysteine had the most marked action.

Experiments were then carried out to determine whether the addition of cysteine *etc.* to brain tissue already exposed to iodoacetic acid for $2\frac{1}{2}$ hours, would restore the normal rate of O_2 uptake in the presence of glucose. The results were negative showing that the alleviating action of the sulphur compounds when added together with the iodoacetic acid was due to direct interaction with the halogen acid.

Some evidence for the interaction of thioglycollic acid with iodoacetic acid has been obtained by Bersin [1932].

These experiments demonstrate the lability of the halogen atom of iodoacetic acid. The comparative ease with which it combines with thiol groups would lead one to consider the possibility that the physiological activity of iodoacetic acid may be connected with an interaction with naturally occurring thiol groups in the living cell. If this were so, it would be anticipated that thiol compounds may play a part in some of the biochemical reactions specifically affected by iodoacetic acid.

In view of the fact that fermentation of glucose by yeast is inhibited by small concentrations of iodoacetic acid [*cf.* Lundsgaard, 1932] experiments were undertaken to determine whether thiol compounds play any rôle in the process of glucose fermentation by yeast.

B. Experiments with yeast.

Technique.

In the experiments to be described the Barcroft differential manometer was used. Observations were made of the O_2 uptake and CO_2 output by yeast in the presence of phosphate buffer solution of p_H 6·0 containing 1·6 % glucose. Two manometers were used for the estimations. In the first manometer 0·3 cc. 20 % KOH was placed in the inner tube of the reaction vessel, the O_2 uptake alone of the yeast being obtained. In the second manometer potash was omitted from the reaction vessel, the readings obtained representing differences between CO_2 output and O_2 uptake. The CO_2 output was determined from the readings of the two manometers. It is important in this work that the p_H of the fluids in the vessels of the manometers should remain the same throughout the experimental period. Using $M/15$ phosphate buffer of p_H 6·0 it was found that no appreciable difference in p_H occurred.

The effect of a thiol compound on the metabolism of yeast was obtained either by adding the substance to the yeast at the commencement of an experiment or by adding it to the yeast after fermentation had proceeded for some time. Actually it was found that the effects of the thiol compound were the same by either method.

Experiments were carried out at 37°, air being present in the Barcroft

manometer vessels. Readings were taken after preliminary shaking for 15–30 minutes to ensure temperature equilibrium and steady rates of O_2 uptake and CO_2 output.

Most of the work so far has been carried out with fresh baker's yeasts—two Belgian varieties and one Dutch (Delft) being used.

The exact procedure was as follows.

In the right-hand vessels A, B, C, D of four Barcroft differential manometers were placed:

(A) 1 cc. *M*/5 phosphate buffer solution p_H 6·0, 1 cc. saline, 0·5 cc. 10 % glucose solution (in saline), 0·5 cc. 2 % yeast suspension (in saline); no potash was present;

(B) as in (A), but potash was placed in the inner tube;

(C) as in (A), with the exception that 1 cc. saline was replaced by 0·5 cc. saline and 0·5 cc. of 0·5 % solution of thiol compound (in saline); no potash was present;

(D) as in (C), but potash was placed in the inner tube.

The left-hand vessels each contained 3 cc. saline. Readings were taken every 15 minutes over a period of 3 hours. At the close of the experiment p_H estimations were made to determine whether material changes of p_H had occurred. As a rule no difference in p_H greater than 0·2 was observed.

Results with cysteine.

The rate of CO_2 evolution is slightly greater than the rate of O_2 uptake with all specimens of baker's yeast used, under the experimental conditions described.

The addition of cysteine to the yeast entirely changes the rates of CO_2 output and O_2 uptake. There is an enhanced rate of production of CO_2 and a greatly diminished rate of O_2 uptake.

The results in Table II are typical of cysteine (a Belgian yeast being used). 2·5 mg. cysteine hydrochloride (neutralised with NaOH) were present.

Table II.

		Time (in mins.)			
		30	60	90	150
No cysteine present.	O_2 uptake, mm.^{3}	290	513	753	1204
	CO_2 output, mm.^{3}	334	582	847	1355
Cysteine present.	O_2 uptake, mm.^{3}	79	148	209	308
	CO_2 output, mm.^{3}	414	762	1078	1664

Similar results were obtained with Delft baker's yeast. It is evident that cysteine exercises a considerable inhibitory action on the O_2 uptake of yeast in the presence of glucose. This result is quite different from that obtained with brain tissue where cysteine has relatively little inhibitory action on the oxygen uptake in the presence of glucose (Table I).

The effect of cysteine on the O_2 uptake and CO_2 output resembles that of cyanide, with the exception that the inhibitory effect of cyanide on yeast respiration is much more pronounced.

The increase in the rate of CO_2 formation by the cysteine only occurs under aerobic conditions. Under anaerobic conditions or in the presence of cyanide cysteine does not increase the rate of CO_2 formation—it has on the contrary a small inhibitory action. Thus, in one experiment, in the presence of 0·015 % HCN the evolution of CO_2, under the conditions given above (and using a Belgian yeast), was 503 mm.^{3} in 60 mins. and 930 mm.^{3} in 120 mins. In the presence of 0·015 % HCN and 0·15 % cysteine the evolution of gas was 459 mm.^{3} in 60 mins., and 821 mm.^{3} in 120 mins., indicating some toxicity of cysteine to the fermenting complex.

Results with glutathione.

The action of glutathione is similar to that of cysteine in bringing about a considerable change in the relative rates of CO_2 output and O_2 uptake. Whereas, however, cysteine greatly diminishes the O_2 uptake of yeast in the presence of glucose, glutathione has a relatively small effect.

The following results (Table III) are typical for glutathione (a Delft yeast being used).

Table III.

		Time (in mins.)		
		30	60	90
No glutathione added.	O_2 uptake, mm.^{3}	314	577	833
	CO_2 output, mm.^{3}	414	761	1120
2·5 mg. glutathione added.	O_2 uptake, mm.^{3}	293	512	702
	CO_2 output, mm.^{3}	534	1149	1784

Glutathione brings about a gradual decrease in the rate of O_2 uptake and a decided increase in the rate of CO_2 output. Moreover the rate of CO_2 output appears to increase with time in presence of added glutathione.

Preliminary experiments have indicated that the greater the quantity of glutathione added, the greater is the rate of evolution of CO_2, the limiting rate being equal to that obtained when glucose is fermented under anaerobic conditions (*i.e.* in nitrogen and in the absence of added SH compounds[1]). Thus the ratios of the rates of formation of CO_2 with baker's yeast in the presence of 1·6 % glucose in nitrogen, in air and in the presence of $M/400$ glutathione and air were 100 : 60 : 94.

Effect of cysteine on fermentation of fructose.

Cysteine behaves in the same way towards the fermentation of fructose as towards that of glucose.

The following results (Table IV) were obtained using 0·5 cc. of a 5 % suspension of a baker's yeast, the conditions being those described for glucose. 2·5 mg. cysteine hydrochloride (neutral) were added.

[1] The effect of glutathione varies quantitatively with the type of yeast. The tripeptide was found to enhance the rate of CO_2 formation with a Delft baker's yeast at concentrations which were ineffective with a Belgian yeast.

Table IV.

		Time (in mins.)			
		30	60	90	120
No cysteine present.	O_2 uptake, mm.^{3}	547	1055	1532	1942
	CO_2 output, mm.^{3}	597	1189	1837	2499
Cysteine present.	O_2 uptake, mm.^{3}	77	134	189	211
	CO_2 output, mm.^{3}	864	1726	2584	3341

Effect of cysteine on respiration of yeast in presence of glycerol.

The action of cysteine on the respiration of yeast in the presence of glycerol (0·5 cc. of 10 % solution) was investigated. The following results (Table V) were obtained, using 0·5 cc. of a 5 % suspension of baker's yeast.

Table V.

		Time (in mins.)		
		30	60	90
Cysteine absent.	O_2 uptake, mm.^{3}	150	279	391
	CO_2 output, mm.^{3}	100	191	273
4 mg. cysteine added.	O_2 uptake, mm.^{3}	133	244	336
	CO_2 output, mm.^{3}	113	214	298

It will be observed that cysteine had a relatively small effect either on the CO_2 output or on the O_2 uptake of yeast in the presence of glycerol.

It is evident from these results with glycerol and from the lack of a marked inhibition of O_2 uptake by brain in the presence of glucose that the inhibitory effect of cysteine on hexose oxidation by yeast is intimately associated with a specific process occurring in the breakdown of the hexose molecule by yeast.

Mode of behaviour of cysteine and glutathione.

It is apparent from the results cited that thiol compounds as represented by cysteine and glutathione affect markedly the relationship between the oxidation and the fermentation of glucose by yeast. The liberation of CO_2 approaches the rate obtained when yeast ferments glucose under anaerobic conditions. Whereas, however, the O_2 uptake by yeast in presence of glucose is greatly inhibited by cysteine, glutathione has but a small effect. The phenomena observed with these thiol compounds are similar to those described by Warburg [1926] in his studies of the relative effects of cyanide and carbylamine on the Pasteur reaction. Carbylamine intensified aerobic fermentation without any marked action on the respiration, whilst cyanide greatly decreased the respiration.

As Warburg has pointed out, many substances, *e.g.* narcotics, will affect the Pasteur reaction, the normal relationship between aerobic fermentation and respiration being disturbed. Glutathione, however, is a substance which occurs naturally in the living cell and hence results with it must have some significance in the dynamics of normal cell processes.

Warburg [1926] suggests that the behaviour of carbylamine in affecting aerobic fermentation but not respiration is due to two properties of the sub-

stance: an ability to enter into complex combinations with heavy metals and an inability to react with the heavy metal of the respiration ferment. The latter is more sensitive towards free cyanide than the catalyst of the Pasteur reaction.

Perhaps the effects of cysteine and glutathione may be interpreted in a similar manner. Cysteine is able to enter into combination both with the catalysts of respiration and of the Pasteur phenomenon, whilst glutathione can only affect the latter. This explanation, however, is not very satisfactory, for the marked inhibitory effect of cysteine on the rate of respiration in the presence of glucose is not found to occur with the respiratory rate in the presence of glycerol. If cysteine enters into combination with a respiratory catalyst it would be anticipated that all oxidations of yeast would be affected. The conclusion would be that either there is a particular catalyst which comes into play during glucose breakdown and which is affected by cysteine and not by glutathione, or that there is a particular process occurring in the aerobic breakdown of glucose by yeast whose course is affected to a much greater extent by the reducing action of cysteine than by that of glutathione. Further investigation is necessary to settle this problem.

Bumm and Appel [1932] have demonstrated that glutathione increases aerobic glycolysis by tumour cells to the limits found anaerobically, and it seems likely that this effect is closely related to the phenomenon already described.

The inference would be that glutathione, present in the living cell, plays some controlling part in the relationship between respiration and fermentation (or glycolysis) in the presence of glucose.

Preliminary experiments with brewer's yeast have shown a behaviour with glutathione similar to that experienced with baker's yeast, but the effect is not so well marked. This is to be correlated with the fact that the respiration of the brewer's yeast was found to be extremely small compared with the fermentation. Moreover, iodine titrations showed a larger content of —SH groups in the brewer's yeast than in the baker's yeast. The addition of an extract of brewer's yeast to baker's yeast was found to increase the rate of aerobic fermentation of glucose, this being probably due to glutathione present in the extract.

The fact that cysteine or glutathione has the property of securing a rate of fermentation by yeast which approximates to that obtained under anaerobic conditions recalls the observation [Quastel and Stephenson, 1926] that thiol compounds enable a strict anaerobe such as *B. sporogenes* to proliferate under aerobic conditions. The suggestion made then was that the presence of thiol compounds established a limiting reduction potential which was necessary for proliferation of this organism. In an analogous manner it may be suggested that the Pasteur phenomenon—the relationship between aerobic fermentation and respiration—is associated in the cell with the existence of a particular reduction potential, whose magnitude will depend in part on the presence of

thiol compounds. Whether this be the case or not there seems little question that thiol compounds normally play some part in determining the relationship between fermentation and respiration of yeast in the presence of glucose.

Further work on this question is now in progress.

SUMMARY.

1. Iodoacetic acid reacts with sodium thiosulphate, cysteine and glutathione. These substances diminish the toxicity of iodoacetic acid towards biological processes.

2. Cysteine and glutathione markedly affect the relationship between fermentation and respiration of baker's yeast in the presence of glucose.

3. Cysteine inhibits the oxygen uptake of baker's yeast in the presence of glucose; glutathione has but little effect. Both thiol compounds increase the rate of CO_2 formation, glutathione having the greater action.

4. The fermentation of fructose is affected in a similar manner to that of glucose by cysteine.

5. The O_2 uptake and CO_2 output of yeast in presence of glycerol are only slightly affected by cysteine.

6. The controlling action of glutathione on aerobic fermentation is probably of significance in the normal metabolism of the cell.

We wish to express our thanks to the Medical Research Council for a grant towards the equipment of this laboratory, and for a whole time assistance grant to one of us.

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CCLVII. THE ANTISCORBUTIC POTENCY OF APPLES. V.

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(*Received November 7th, 1932.*)

CULTIVATED varieties of apples fall into two groups as regards the chromosome number of their nuclei, the so-called diploids with 34 and triploids with 51 chromosomes respectively. Preliminary experiments [Crane and Zilva, 1931] have shown that triploid apples reveal a tendency to possess a higher vitamin C content than diploids. The number of varieties tested in the experiments mentioned was, however, small and it was felt desirable to extend it. In this communication the results of tests carried out with a further number of triploid varieties are given and are compared with those previously obtained with triploid and diploid apples.

EXPERIMENTAL.

The following are the cultural details of the varieties tested.

Gennet Moyle and Ribston (*East Malling*).

We wish to thank Mr R. G. Hatton and Mr A. C. Painter of the East Malling Research Station, East Malling, Kent, for kindly offering this material and for supplying the cultural details connected with it.

(*a*) Age of trees—9 years.

(*b*) Stock—No. 5 (Doucin Amélioré).

(*c*) Soil—light loam over Kent ragstone—known as Hythe beds (lower greensand series).

(*d*) Manurial treatment—early years alternate dressings of London stable manure and shoddy. Later years sulphate of potash 3 cwt. per acre *plus* 6 cwt. meat and bone meal. In 1931 received 3 cwt. of sulphate of potash per acre only.

Reinette du Canada.

The bulk of this material was obtained from the Royal Horticultural Society's Gardens, Wisley, Ripley, Surrey, and we wish to thank Mr R. L. Harrow and Mr F. J. Chittenden for their help in supplying it and for the following cultural details in connection with it.

(*a*) Age of trees—approximately 25 years.

(*b*) Stock—crab stock.

(*c*) Soil—light loam over gravel.

(*d*) Manurial treatment—dressing of superphosphate of lime in the spring at the rate of approximately $\frac{1}{4}$ lb. to the square yard. Garden refuse and a dressing of sulphate of potash (2 oz. to the square yard) were forked in in 1930.

(*e*) Crop—"moderate."

[1] Member of the Scientific Staff of the Medical Research Council working with grants for assistance and expenses.

As the quantity of material obtained from this source was not quite sufficient to complete the experiment, a further small supply was obtained from a nursery at Slough, Bucks. The cultural details for this material are as follows:

(*a*) Age of trees—5 years.
(*b*) Stock—English broadleaf.
(*c*) Soil—heavy loam.
(*d*) Manurial treatment—liberal stable manure.
(*e*) Nature of crop—generally medium.

Warner's King.

The material was obtained from a fruit farm in Kent. The cultural details are as follows:

(*a*) Age of trees—very old trees severely pruned.
(*b*) Stock—probably seedling crabs.
(*c*) Soil—medium light soil on ragstone.
(*d*) Manurial treatment—sheep-cropped but inclined to be in starved condition. Annual application of about 4 cwt. of potash salts.

Ribston (Canadian); Baldwin 1931 *and Gravenstein* 1931.

We have to thank Dr W. S. Blair of the Dominion Experimental Farm, Kentville, Nova Scotia, Canada, for this material and for the following cultural details:

(*a*) Age of trees—planted in 1912.
(*b*) Stock—French stock imported from France. Propagated in Nova Scotia at a local nursery in 1909.
(*c*) Manurial treatment—the trees since coming into bearing have been supplied with approximately a 5–9–7 fertiliser. This was used at the rate of 5 lbs. per tree in 1924, 1925 and 1926 and 7 lbs. per tree in 1927 and 1928. The fertiliser after 1928 was increased in nitrogen and a fertiliser made up of 200 lbs. of nitrate of soda, 200 lbs. of sulphate of ammonia, 300 lbs. of superphosphate and 100 lbs. of muriate of potash was applied at the rate of 8 lbs. per tree and this mixture was used at the same rate in 1930 and 1931. No farmyard manure was used. This was applied broadcast about the 10th of May, extending about 3 ft. beyond the area of the branches and on the grass as well as on the cultivated areas.
(*d*) Soil—sandy loam. These trees were clean cultivated until 10 years of age and then a strip of grass was left under the tree, varying from 2 to 8 ft. wide with trees in centre of strip, until the present. This strip of grass was clipped 2 to 3 times during the season, grass was not more than 6 ins. in height at any time. The cut material was evenly spread as a mulch under the trees. The area between this and the next tree was cultivated. The ground was fall ploughed. This cultivation was continued to July 1st at intervals of 2 weeks from the 8th May.

The rows are 40 ft. apart, every other tree is a Wagner. The above varieties are 40 ft. each way with a Wagner filler between, making all trees 20 by 40 ft.

Baldwin 1930.

One barrel ("grade 1") of this material was obtained from the United Fruit Co., Nova Scotia. No cultural details were obtained.

Gravenstein 1930.

One barrel ("grade 1") of these apples was obtained from the United Fruit Co., Nova Scotia, at the same time as the Baldwin 1930 material. In this case also no cultural details were available.

The vitamin C tests were carried out by the procedure already described [Bracewell, Hoyle and Zilva, 1930], except that owing to shortage of experimental material animals dying from intercurrent diseases could not be replaced. We are, however, of the opinion that the assessment of the degree of protection from scurvy in these cases did not suffer much in accuracy. Tables I, II and III set out the origin, the chromosome number and antiscorbutic potency respectively of the above varieties as well as of the varieties previously tested.

Table I.

Variety	Parentage	Origin
Bramley's Seedling	Not known	Raised by Mr Bramley of Southwell, Notts. Introduced in 1876
Belle de Boskoop	,,	Originated at Boskoop in Holland in 1856
Gennet Moyle	,,	Not known
Reinette du Canada	,,	Uncertain. Known in France in the eighteenth century
Blenheim Orange	,,	Raised at Woodstock near Blenheim by Mr Kempster. Introduced about 1818
Warner's King	,,	Found at Weavering in Kent at the end of the eighteenth century
Ribston Pippin	,,	Raised at Ribston Hall, Knaresborough, about 1709, from seeds brought from Rouen
Baldwin	,,	Originated about 1740 as a chance seedling on the farm of Mr Ball, Wilmington, Massachusetts
Gravenstein	,,	Said to have been found at the castle of Grafenstein, in Schleswig-Holstein. Introduced about 1760
Lane's Prince Albert	,,	Found in a garden at Berkhampstead and introduced by Messrs Lane of that town in 1857
Newton Wonder	,,	Raised by a Mr Taylor of Kings Newton, Derby, and introduced by Messrs Pearson and Co. about 1887
Cox's Orange Pippin	Alleged to be a seedling from Ribston Pippin	Raised about 1830 by a Mr Cox of Colnbrook Lawn, near Slough, and introduced by Mr Chas. Turner
King Edward VII	Said to be Blenheim Orange × Golden Noble	Introduced by Messrs Rowe of Worcester about 1908
Worcester Pearmain	Alleged to be a seedling from Devonshire Quarrenden	Originated at Swan Pool, near Worcester, by a Mr Hale. Introduced by Messrs Smith of Worcester in 1874

Table II.

	Chromosome numbers ($2n$)				
Variety	Kobel	Crane and Lawrence	Nebel	Darlington and Moffett	Rybin
Bramley's Seedling	—	51	—	51	—
Belle de Boskoop	*ca.* 46	—	51	—	—
Gennet Moyle	—	51	—	51	—
Reinette du Canada	38–40	—	51	—	51
Blenheim Orange	—	51	51	51	—
Warner's King	42	—	—	—	—
Ribston Pippin	42	—	51	51	—
Baldwin	48–49	—	51	51	—
Gravenstein	45–46	—	51	—	—
Lane's Prince Albert	—	34	—	34	—
Newton Wonder	—	34	—	34	—
Cox's Orange Pippin	34	34	—	34	—
King Edward VII	Not cytologically examined but resembles a diploid in characters				
Worcester Pearmain	—	34	—	34	—

Table III.

Daily dose of apples g.		3	5	10	20
Bramley's Seedling.	Triploid	+ + + ⊥	+ + + +	+ + + +	+ + + +
Belle de Boskoop	,,	+ + + ⊥	+ + + ⊥ to + + + +	+ + + +	+ + + +
Gennet Moyle	,,	+ + +	+ + + ⊥	+ + + +	Not tested
Reinette du Canada	,,	+ ⊥	+ +	+ + + ⊥	Not tested
Blenheim Orange	,,	⊥	+ + +	+ + + ⊥	+ + + +
Warner's King	,,	⊥	+ + ⊥	+ + + ⊥	+ + + +
Ribston, East Malling	,,	+	+ ⊥	+ + + ⊥	Not tested
Ribston, Canadian	,,	0 to ⊥	⊥	+	+ + + ⊥
Baldwin 1930	,,	⊥	+	+ + ⊥	+ + + +
Baldwin 1931	,,	0	⊥	+ + +	+ + + +
Gravenstein 1930	,,	0	0	0	⊥
Gravenstein 1931	,,	0	0	0	⊥ to +
Lane's Prince Albert.	Diploid	⊥	+ + +	+ + + +	+ + + +
Newton Wonder	,,	⊥	+ ⊥	+ + + +	Not tested
Cox's Orange Pippin	,,	⊥	+	+ + ⊥	+ + + ⊥
King Edward VII	,,	0	⊥	+ + ⊥	+ + + ⊥
Worcester Pearmain	,,	0	0	⊥	+ + +

Conclusions.

As in the case of the diploid, the triploid apples show a variation in anti-scorbutic activity amongst the varieties tested. Of these at least three, Bramley's Seedling, Belle de Boskoop and Gennet Moyle, stand out as exceptionally active, and four varieties, namely Reinette du Canada, Blenheim Orange, Warner's King and Ribston Pippin are roughly of the same order of potency as Lane's Prince Albert, an outstandingly active variety of the diploid group of apples. The Gravenstein variety, although a triploid, is the least active apple registered in this investigation. It is clear that the results, although suggesting that some connection may exist between the chromosome number and the antiscorbutic activity of the apple, do not supply incontrovertible evidence to that end.

It must however be stressed in this connection that nothing of real value is known of the parentage of the apples used in this investigation, nor is there available any definite knowledge concerning the origin of the triploid form of apple in general. Triploids commonly originate from the crossing of tetraploids with diploids, but since a tetraploid form of the cultivated apple is unknown one is forced to consider the probability that triploid varieties of apples arose from diploids by the accidental functioning of an unreduced gamete. Rybin [1927], moreover, has actually reported triploids arising from diploid parents and, further, triploid forms have arisen in a similar way in other fruits [Osawa, 1920].

It is probable that a triploid derived from "selfing" a diploid with a high vitamin C content or from crossing two very active diploids would yield a triploid variety richer in vitamin C than either diploid parent and that triploids derived from low content diploids would be correspondingly less active. A similar variation in the distribution of other characters among apples is known. Vigour of growth may be cited as an example. Although triploids are

in general more vigorous than diploids this property varies amongst the individual triploid and diploid varieties.

Conclusive evidence could only be obtained by comparing the vitamin C content of triploid forms with that of the diploid apples from which they were derived. This would entail the production of the corresponding triploid forms artificially, a task which is fraught with almost insuperable difficulties. The path for further experiments obviously lies in the direction of other fruits or vegetables in which polyploidy occurs, or can be induced with greater ease, and in which strictly comparable diploid and polyploid forms can be obtained.

We are indebted to Mrs K. M. Balfour for helping us with the tests in this investigation.

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CCLVIII. THE ANTISCORBUTIC ACTIVITY OF THE CORTEX OF THE SUPRARENAL GLAND OF THE OX.

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(*Received November 10th, 1932.*)

In connection with an inquiry which has been going on for some months on the question of the identity of hexuronic acid with the antiscorbutic factor, I took the opportunity of testing the cortex of the suprarenal gland of the ox. This as will be seen from Fig. 1 was more active than decitrated lemon

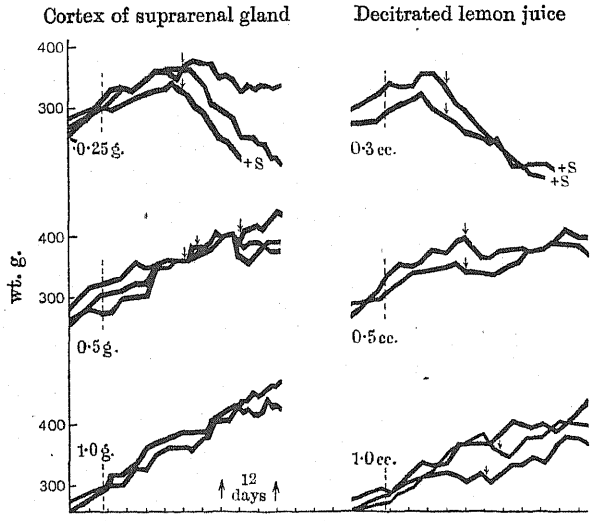


Fig. 1.

↓ = onset of scurvy.
+S = death from scurvy.
⋮ = beginning of administration of dose.

juice tested at the same time and under the same conditions. In view of the fact that Dr L. J. Harris, who was kind enough to show me his detailed results while they were in the press [see also 1932], is independently publishing his experiments on the activity of the cortex of the suprarenal gland, I think it of interest also to record my tests the outcome of which is similar to his.

REFERENCE.

Harris (1932). *Lancet*, ii, 235.

[1] Member of the Scientific Staff of the Medical Research Council working with grants for assistance and expenses.

CCLIX. PHYSICO-CHEMICAL STUDIES OF LIPOIDS IN THEIR RELATIONS TO SALTS, DRUGS AND PROTEINS[1].

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(*Received October 18th, 1932.*)

Although extensive investigations [Remesow, 1930] have been made recently on the physico-chemical behaviour of some lipoid sols, explanations are still lacking of some problems interesting to the biologist. Of these problems, the specific therapeutic effect of bromides and the relation of lipoids to proteins have been chosen for a physico-chemical investigation.

In a previous paper [Spiegel-Adolf, 1932], an *in vitro* analogy with the specific effect of bromides on the substance of the central nervous system was demonstrated. While the Hofmeister series of anions gives no clue in this direction, it could be shown that bromides have a stronger depressing effect on the viscosity of lecithin sol than any other salts, except iodides containing traces of free iodine. This seemed to suggest that the specific effect of bromides is not due to the bromide ions, but to free bromine. Pauli [1929] has pointed out the possibility of such a mechanism, and recent work of Lanza [1931] has proved that the addition of iodine to KI does not change the molecular concentration and only very slightly affects the electrolytic dissociation of the latter. Owing to the unsaturated fatty acid content of lecithin a reaction with bromine or iodine is possible. As there is chemical evidence that fatty acids add KI, too, and are then enabled to bind more of other substances, even the behaviour of the compounds of lecithin and protein can be explained in this way.

In order to test this hypothesis, the following series of experiments was made. Though egg-lecithin (Merck) is, according to Remesow [1930], far from pure, it was used, since most work has been done on it, and results permitting comparison with the latter were needed. The lecithin sol was made according to Keeser [1924] by adding small portions of boiling alcoholic solution of lecithin to boiling redistilled water. Some figures concerning the conductivity and c_H of such sols are reported in Table IV. In order to check the possible influence of traces of alcohol, emulsions of lecithin in redistilled water were used in several experiments. Such emulsions [*cf.* Thomas, 1915], are more opaque than a fresh sample of a sol made after Keeser, but are nevertheless stable. The protein used consisted of serum-albumin and pseudoglobulin prepared from horse-serum by $(NH_4)_2SO_4$ fractionation, and purified by reprecipitation, dialysis and electrodialysis.

[1] Partly read before the Physiological Society of Philadelphia, March 14th, 1932.

It is known that the total refraction of a solution is equal to the sum of the refractions due to the separate solutes, but this is only true if there are no chemical reactions between them. If KBr and KI containing free iodine should act on the lecithin in the way suggested above, it is to be expected that the refractive index of a mixture of lecithin sol with such salts will differ from that of the same sol with KCl, for instance. As only very slight changes were expected, the interferometer of Zeiss was used for the following investigations [*cf.* Hirsch and Kunze, 1922]. As the lecithin sol that was to be used was far from pure, and the molecular weight of the colloidal particle unknown, no attempt was made to obtain absolute values for the refraction. The 1 % lecithin sol was diluted 20 times; the final concentration of the salts was 0·01 *M*. The interferometric values were measured in the lecithin sol, the salt solution and in a mixture of lecithin sol and salt. Correction for water was made in every case. The results are given in Table I.

Table I. *1/20 lecithin sol—74* I.U., *cell 20 mm.*

Neutral salt concentration	KCl		KBr		KI		KI+I		KCNS	
	obs.	calc.	obs.	calc.	obs.	calc.	obs.	calc.	obs.	calc.
0·01 %	200	199	252	238	347	339	367	349	288	284
Difference	1		14		8		18		4	

In all cases the sum of the refractions of the separate components was lower than the observed value. The effect of bromide however was by far the greatest, only being surpassed by the effect of KI containing 0·02 g. of iodine per 10 cc. of fresh *N* KI. KCl did not significantly alter the refraction, even when the concentration of the salt was raised 10 times. The absolute values of the changes in interferometric units (I.U.) are only small, but while it is possible to get exact readings to ± 1 I.U., there is, according to Marc [1912], less danger, for colloids at least, of being mistaken in the right band when using dilute solutions; in fact the readings permitted no doubt in this direction. On the other hand, the use of more concentrated solutions would not increase the sensitivity of the method, as the increased opacity would necessitate a shorter cell. The absolute values are well within the ranges that could be expected according to other findings [Hirsch and Kossuth, 1922].

As was mentioned in the previous paper, the swelling of lecithin sol is lowered to very different degrees by chloride and bromide. It seems that a part of the lowering effect on the viscosity common to both salts is due to electrical discharge of the lecithin sol, hydration being in close connection with the electrical charge. But the greater effect of bromide may be due to colloidal changes consequent on chemical reaction. In order to decide whether the observed relations between viscosity and refraction hold for other colloids, interferometric measurements were made on protein solutions in the same way as on the lecithin sol, and it was found that the two salts had practically

the same effect on the viscosity and the refraction of both serum-albumin and pseudoglobulin.

The experiments which are described in this paper confirm those previously reported, in demonstrating the specific effect of bromides. Naturally they are not sufficient to decide whether bromides act in the same way on the brain, or if they do, that this physico-chemical behaviour is the reason for their specific effect.

Winterstein [1926], considering the results of Hansteen Cranmer confirmed by Biedermann, believes that the lipoids actually present in the cells are quite different from those obtained by extraction. On the other hand, Handovsky and Wagner [1911] observed no reaction between lecithin sol and drugs in their experiments on viscosity. In order to investigate the possibility of an additional parallelism between viscosity and refraction, further experiments were made on these lines. As far as possible, the kind and concentration of the drugs used were the same as those employed by Handovsky and Wagner. Experiments were also made with sodium-luminal, the clinical use of which is similar to that of bromides. Finally, a convulsion-producing poison, strychnine nitrate, was tried in the same way. The results are summarised in Table II.

Table II. *1/20 lecithin sol—74* I.U.

1% lecithin sol diluted %	Final concentration	Interferometric units			
		Measured	Calculated	Difference	Cell mm.
1/20	0·1 % sodium-luminal*	288	271	17	20
1/20	0·15 % chloral hydrate	271	255	16	20
1/10	Sulfonal, half saturation	102	103	−1	5
1/10	Sulfonal, 9/10 saturation	137	137	0	5
1/10	Trional, half saturation	146	146	0	5
1/10	Trional, 9/10 saturation	208	211	−3	5
1/10	0·05 % urethane	302	302	0	5
1/10	0·01 % strychnine nitrate	98	98	0	5

* NaOH of the same p_H as the sodium-luminal solution has no effect on the refraction of the lecithin sol.

The figures suggest a reaction between lecithin sol and sodium-luminal, or chloral hydrate, though they may also mean that the dispersion of the lecithin is increased. The negative results with sulfonal, trional and urethane agree with the findings of Handovsky and Wagner. The opposite effect exercised by the highest concentration of trional on lecithin sol, is explained by a decrease of the dispersion of the latter. Strychnine nitrate had no effect on the refractive power of lecithin sol.

The reaction between lecithin and proteins depends largely upon the c_H of the solution, the presence of neutral salts, and the age of the lecithin sol. The first point has been extensively investigated by Rona and Deutsch [1926], but underestimation of the important rôle of salt concentration in the relation between lecithin sol and proteins seems to be the reason for contradictory opinions in this matter.

It was noted previously [Spiegel-Adolf, 1932] that electrolyte-free solutions of serum-albumin and pseudoglobulin flocculate lecithin-sol made according to the method of Keeser [1924]. In good accord with results found by Pauli and Weiss [1928] in their investigations on colloid dyes, pseudoglobulin has a stronger flocculating effect than serum-albumin. More than 50 times as much of either protein is necessary for flocculation of 24 hours-old lecithin sol than for a week-old sol. Table III records the results of some experiments of this kind.

Table III.

× = flocculation.

Concentration of protein %		1·5	0·75	0·28	0·14	0·045	0·028	0·014	0·005	0·003
Fresh lecithin sol	Serum-albumin	× × ×	0	0	0	—	0	0	0	—
	Pseudoglobulin	× ×	× ×	×	0	—	0	0	0	—
Old lecithin sol	Serum-albumin	× × ×	× × ×	× × ×	× × ×	0	0	0	0	—
	Pseudoglobulin	× × ×	× × ×	× × ×	× × ×	—	× ×	× ×	×	0

According to Freundlich [1922], the ageing of lecithin sol consists of changes by which the latter partly loses its hydrophile properties and grows more hydrophobe. In good accord with this, the amounts of protein necessary for the flocculation of aged lecithin sol are within the range of the corresponding values for gold sol [Spiegel-Adolf, 1927].

There seem to exist no systematic investigations of the physico-chemical changes during ageing of lecithin sol, though there are various hints in this direction [Porges and Neubauer, 1908; Rona and Deutsch, 1926]. For the better understanding of what has been said above and what is to follow, some observations are recorded in Table IV.

Table IV.

Age of lecithin sol (Keeser)	Conductivity rec. Ohm	Viscosity 1/5 sec.	c_H
24 hours	$1{\cdot}69 \times 10^{-5}$	1405	$1{\cdot}37 \times 10^{-4}$
8 days	$1{\cdot}84 \times 10^{-5}$	1380	—
12 days	—	—	$1{\cdot}77 \times 10^{-4}$
18 days	$2{\cdot}10 \times 10^{-5}$	—	$1{\cdot}95 \times 10^{-4}$
30 days	$2{\cdot}33 \times 10^{-5}$	—	—
Lecithin sol water emulsion 24 hours	—	—	$1{\cdot}95 \times 10^{-4}$

The above experiments were performed on lecithin sols kept exclusively in bottles of pyrex glass under optimum conditions for stability. Nevertheless, the 1 % solutions, which were first translucent and of a reddish-yellow hue, became more opaque after a few days and, in the course of time, much of their colour faded. A sample kept for a year was nearly white. If infection by moulds were avoided, the sol seemed to be fairly stable. As long as no precipitation had occurred, no changes in the refractive index of the colloid solution could be detected, and measurements of the c_H using the hydrogen electrode showed only small changes. Measurements made on the same sample immediately after preparation, and a week later, indicated a slight increase of the acidity.

After 18 days, a c_H was reached corresponding to that of a fresh watery emulsion of lecithin. The secondary increase of c_H was accompanied by a similar change of the conductivity, while the viscosity showed a decrease from the initial value.

Porges and Neubauer [1908] have already shown that the flocculation of lecithin sol by proteins is inhibited by the presence of neutral salts. This is not a property peculiar to the lecithin sol, but is observed with negative sols such as colloid solutions of gold, mastic and cholesterol. In this way, it is possible to obtain stable solutions containing known amounts of lecithin, protein, and salts. In a previous communication [1932], the viscosity of such solutions under the influence of chlorides and bromides has been investigated. Handovsky and Wagner [1911] had used similar solutions in order to ascertain whether there were any reason for assuming the formation of a compound between protein and lecithin sol, but obtained negative results. Later on, Arnd and Hafner [1926] found that the refractive power of a pseudoglobulin solution was markedly lowered by the addition of lecithin sol, which they accepted as proof of formation of a lipoid-protein compound. In order to explain the discrepancy between these opinions, experiments were started, using interferometric measurements. At the same time, the influence of different salts was tested. Some results are reported in Tables V and VI.

Table V. *Lecithin sol and protein (final concentration 0·1 %).*

NaCl concentration *N*	Serum-albumin		Pseudoglobulin	
	Observed	Calculated	Observed	Calculated
0·025	162	160	137	146
0·05	—	—	222	220
0·1	398	396	366	365
0·2	669	668	—	—

Table VI. *Pseudoglobulin and lecithin sol (final concentration 0·1 %).*

0·1 *N* final concentration	KCl		KBr		KI		KCNS		$(NH_4)_2SO_4$	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
Sol 7 days	408	413	527	524	779	776	130*	130*	88*	86*
Sol 14 days	—	—	95*	111*	—	—	—	—	—	—
Fresh 1 day	—	—	528	525	—	—	—	—	—	—

* All measurements were made in a 5 mm. cell except those marked with asterisks, for which a 1 mm. cell was used.

The figures suggest the following interpretation. As long as the salt concentration is sufficient to avoid the faintest signs of flocculation, no changes of the refractive indices are observed. Different salts seem to behave in the same way. If the salt concentration is lowered to a point at which the solution becomes unstable, then the interferometric value of the solution is lower than the sum of the corresponding values for the separate components. The results are the same whether the instability be due to an initial low salt concentration, or whether through the ageing of the colloid, the salt concentration becomes

insufficient. These findings explain the observations of Handovsky and Wagner as well as those of Arnd and Hafner [1926], though they do not necessarily confirm the conclusions of the latter authors. The decrease in refraction must be explained primarily by a decrease of soluble material.

The first row of figures in Table V indicates a difference between the behaviours of serum-albumin and pseudoglobulin towards lecithin at the same low salt concentration. While the latter is not sufficient to avoid flocculation of the pseudoglobulin-salt-lecithin mixture, it is still able to guarantee the stability of a solution in which pseudoglobulin is replaced by serum-albumin. Although there is some similarity in this respect to the behaviour of the same salt-protein mixtures toward gold sol, it is not possible to speak, in the case of lecithin sol, of the protective power of the proteins. The same amount of neutral salt which inhibits the flocculating effect of proteins, in both cases precipitates the gold sol, but leaves the lecithin sol apparently unchanged. In earlier experiments on gold sol, evidence has been found for assuming that a compound of proteins and neutral salt would be able to protect the gold sol from the flocculating effect of further salt addition. Experiments of a similar kind were started with lecithin sol, only, instead of salt addition, pseudoglobulin was tried for testing the protective power of a serum-albumin-neutral salt compound. Only in cases in which there was a certain gap between the salt concentration able to inhibit the flocculating effect of serum-albumin and that having the same influence on pseudoglobulin, are such experiments possible. For this purpose, only lecithin sol of a certain age could be used. In recently prepared samples, the differences are too slight; in very old sols, sometimes the protective power of salts, even against albumin, vanishes. Only lecithin sols 14–21 days after the preparation according to Keeser, fresh emulsions of lecithin in distilled water, or lecithin sols derived from an older, perhaps oxidised sample of lecithin, proved to be of use for the experiments in question. The results of two series are summarised in Table VII.

These results seem to show that under certain conditions, flocculating concentrations of pseudoglobulin do not show this effect in the presence of serum-albumin and neutral salt. Though the latter at the same concentration is, in itself, not sufficient to prevent the flocculation effect of pseudoglobulin, its presence is necessary for giving the serum-albumin protective power. Non-flocculating concentration of the latter in an electrolyte-free medium is unable to inhibit the precipitation of the lecithin sol by pseudoglobulin. The negative result of interferometric investigation made in order to trace some reaction between electrolyte-free serum-albumin and pseudoglobulin was in accordance with these findings. For this reason, the above-mentioned influence of the neutral salt-serum-albumin compound on pseudoglobulin cannot be directly compared with observations made by Pauli and Singer [1932], who found that ovalbumin prevented, to some extent, the flocculation of certain colloid dyes by serum-albumin in the absence of salt.

Next to the salt concentration, the protective power depends upon the

Table VII. *Part I.*

× = flocculation. + = opacity.

Lecithin sol 19 days old	NaCl final concentration *N*	Serum-albumin %	Pseudoglobulin %	Result
1/4	0·05	0	0	0
1/4	0	0·25	0	× × ×
1/4	0	0	0·25	× × ×
1/4	0·05	0·25–0·5	0	0
1/4	0·05	0	0·25–0·5	× × ×
1/4	0·05	0·25	0·25	0
1/4	0·05	0·2	0·3	0
1/4	0·05	0·15	0·35	× ×
1/4	0·05	0·1	0·4	× × +

Table VII. *Part II.*

Lecithin sol aged by boiling	KCl final concentration *N*	Serum-albumin %	Pseudoglobulin %	Result
1/4	0·1	0	0	0
1/4	0	0·75	0	× × ×
1/4	0	0	0·75	× × ×
1/4	0·1	0	0·7–1·4	×
1/4	0·1	0·75–1·4	0	0
1/4	0·1	0·75	0·7	0
1/4	0·1	0·6	0·84	0
1/4	0·1	0·45	1·0	0
1/4	0·1	0·3	1·1	× + +
1/4	0·1	0·15	1·25	× × ×

ratio of serum-albumin to pseudoglobulin. If the latter falls below 2/3–1/2, precipitation starts. It seems therefore that the behaviour of the total protein mixture toward the lecithin sol depends on the relation of its components. The similarity of this behaviour to that of serum under various pathological conditions is somewhat striking. There, too, the so-called albumin/globulin quotient [Hofmann, 1883] seems to determine, in many respects, the properties of the serum and other biological fluids. The reactions of spinal fluid toward gold sol seem in particular, according to Schmitt [1927] to depend largely on this property. An increase of globulin has been described in nearly all luetical changes. The above-mentioned results may be of some value for a further understanding of luetical reactions using the flocculation methods.

Summary.

1. Confirming prior experiments on viscosity, interferometric measurements seem to give evidence for a supposed reaction between bromine and lecithin sol, but fail to explain the effect of hypnotics.

2. The effect of lecithin addition to proteins depends on the salt content of the latter. In an electrolyte-free medium, flocculation occurs; if there is an excess of salt, no changes can be detected by help of the interferometer. Between those two extremes there are salt concentrations at which lecithin-sol additions cause a decrease of the refractive index of the mixture.

3. For lecithin sols of a given age, different concentrations of neutral salts are required to avoid flocculation by the same amount of serum-albumin or

pseudoglobulin. In mixtures containing both kinds of proteins with just sufficient salt to prevent flocculation by serum-albumin, the serum-albumin-neutral salt compound seems to inhibit to some degree the flocculating power of pseudoglobulin, the final effect depending on the albumin/globulin ratio.

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CCLX. HEXOSEMONOPHOSPHORIC ESTERS: MANNOSEMONOPHOSPHATE.

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It has been established that the hexosemonophosphate formed during alcoholic fermentation of sugars by yeast enzymes is not a single compound but consists of a mixture of aldose and ketose esters.

One, and probably the chief, component of this product was isolated by Robison and King [1931], who concluded from their own experiments and from those of King, McLaughlin and Morgan [1931] that this pure compound most probably had the structure of glucose-6-phosphate. Its specific rotation differed from that first recorded by Levene and Raymond [1930; 1931, 1] for a synthetic ester, which they considered to be glucose-6-phosphate; but the discrepancy largely disappeared as the result of further purification of the synthetic ester [Levene and Raymond, 1931, 2].

Robison and King also isolated a ketose ester similar in most respects to the Neuberg fructosemonophosphate, but having a somewhat lower reducing power (Hagedorn and Jensen); and their further analytical results pointed definitely to the presence of still other esters in the fermentation product. The isolation of one of these esters and the further investigation of the fructosemonophosphate and the residual fractions are described in the present communication.

Isolation of mannosemonophosphate.

During the separation of glucosemonophosphate, many intermediate fractions which no longer contained significant amounts of fructose esters, as judged by the Selivanoff reaction, still gave low values for the iodimetric reducing power. The full value theoretically required for an aldosemonophosphate was obtained only after repeated recrystallisation of the brucine salt from methyl alcohol. The residual fractions obtained in these final stages of the purification were recrystallised 5 times from boiling absolute methyl alcohol and yielded clusters of short needles, very different in appearance from the soft, opaque, silky crystals of pure brucine glucosemonophosphate. These needle fractions were converted into the barium salt, which was purified and analysed by the methods described in previous papers. The results are shown in Table I (salt No. 4), the corresponding values for a typical fermentation hexosemonophosphate (No. 1) and for pure glucosemonophosphate

Table I. *Analyses of barium salts of hexosemonophosphoric acids obtained from the products of fermentation by yeast juice.*

No. of salt	Source of the barium salt	P %	Fructose (Selivanoff) %	Reducing power as glucose (%) H. and J.*	Reducing power as glucose (%) Iodine	Aldosemonophosphate %	$[\alpha]_{5461}^{20°}$	$[\alpha]_{5461}^{20°}$ of free acid
1	Hexosemonophosphate	7·85	6	30·0	25·2	55	+14·4°	+29·5°
2	Glucosemonophosphate	7·86	0·5	35·5	45·7	100	+20·6° (c=0·84 %)	+41·4° (c=0·74 %)
3	Fructosemonophosphate (Neuberg) prepared from highly purified hexosediphosphate	7·80	22	36·2	3·0	7	+ 0·7°	—
4	Brucine salt of new ester recrystallised 5 times from absolute methyl alcohol	7·82	3	36·3	27·5	60	+ 3·1° (c=1·4 %)	— —
5	Residual salt after 17 hours' hydrolysis (28 %) of No. 4 with N HCl at 100°	7·20	1	34·0	25·6	56	+ 3·4° (c=0·4 %)	—
6	Brucine salt from No. 4, recrystallised 5 times from 95 % methyl alcohol	7·82	2	36·9	27·0	59	+ 3·6° (c=0·7 %)	+15·1° (c=1·7 %) +17·0° in 0·1 N H_2SO_4
7	Second fractions from No. 6 recrystallised 4 times from 90 % methyl alcohol and once from water	7·69	1	35·5	30·5	67	+ 3·4° (c=0·7 %)	
8	Another preparation similarly purified	7·65	1·5	36·2	28·0	61	+ 3·1° (c=0·5 %)	
9	Fructosemonophosphate isolated from fermentation hexosemonophosphate	7·73	22	34·9	1·6	3	+ 2·3° (c=0·8 %)	—
10 and 11	Brucine salts obtained in the fractionation of hexosemonophosphate	7·78 7·29	7 4	23·9 20·1	12·6 14·7	28 32	+10·8° +12·7°	—

$C_6H_{11}O_5PO_4Ba$ requires P, 7·85 %; $C_6H_{12}O_6$, 45·6 %.

* Determined with addition of 0·5 cc. of 0·5 N NaOH.

and fructosemonophosphate (Neuberg) being given in the same table for comparison. While the phosphorus content and H. and J. reducing power were the same as for glucose- and fructose-monophosphates, the new compound differed markedly from both these esters in other respects. The iodimetric value was only 60 % of that required for an aldosemonophosphate, but the low Selivanoff value precluded the presence of fructose derivatives in large amount. The low specific rotation also distinguished the new ester sharply from glucosemonophosphate.

Examination by fractional acid hydrolysis. 0·3 g. of this salt, No. 4, was heated with N H_2SO_4 at 100° for 17 hours, the hydrolysis amounting to 28 %; the residual ester was freed from inorganic phosphate and analysed (salt No. 5). The Selivanoff value was still lower (1 %), but the remaining values were not significantly changed from those of the original salt. (The quantity recovered was inadequate for thorough purification and the phosphorus content was somewhat low.)

Further recrystallisation of the brucine salt. The remainder (1·5 g.) of salt No. 4 was reconverted into brucine salt, which was again recrystallised 5 times from 95 % methyl alcohol. Second crops obtained from the mother-liquors were recrystallised 4 times from 90 % methyl alcohol and once from

water at 40°. Analyses of the barium salts prepared from these two fractions are given in Table I (Nos. 6 and 7). No. 8 was a barium salt obtained by similar methods from another hexosemonophosphate preparation.

The properties of these salts suggested that they consisted, wholly or to a very large extent, of a new ester, possibly a mannosemonophosphate.

Hydrolysis of the ester by 0·1 N H_2SO_4.

A quantity equivalent to 1 milligram mol. of the free ester was dissolved in 0·1*N* H_2SO_4 (17 cc.) and heated in a boiling water-bath, the amount of hydrolysis being estimated from time to time. The hydrolysis was stopped after 93 hours, when 79·6 % of the total phosphate had been set free. The solution, which was then dark brown in colour, was neutralised with baryta, treated with twice its volume of alcohol to precipitate the free and combined phosphates and filtered. The filtrate containing the free sugar was evaporated rapidly on a water-bath to a small volume ($p_H < 7·0$), after which the drying was completed in an evacuated desiccator. The residue was extracted repeatedly with boiling 80 % alcohol and the filtered solution evaporated to dryness as before. The rate of hydrolysis and the properties of the sugar produced are shown in Table II.

Table II.

Time (hrs.)	Hydrolysis %	$k \times 10^3$	The syrupy product gave	
4	6·9	0·13	Total P	0·4 mg.
31	43·5	0·13	Hexose (H. and J. reduction)	85 ,,
93	79·6	0·12	Aldose (I reduction)	89 ,,
			$[\alpha]_{5461}$ calculated on hexose	+15°

The rate of hydrolysis was exactly the same as that for the hydrolysis of pure glucosemonophosphate under similar conditions ($k = 0·13 \times 10^{-3}$). Some decomposition of sugar had evidently occurred, but assuming that the decomposition products were optically inactive the specific rotation, calculated on the reducing hexose, was in fair agreement with that of mannose (+ 17·1°).

The sugar solution on treatment with pure phenylhydrazine dissolved in acetic acid gave at once, in the cold, a crystalline derivative; this was recrystallised twice from 80 % ethyl alcohol. The crystals closely resembled those of mannosephenylhydrazone, which was prepared for comparison. They melted[1] at 200–201°; the specimen of mannosephenylhydrazone also melted at 200–201°, as did an intimate mixture of the two substances. Beilstein, Supplement I, gives the M.P. of mannosephenylhydrazone as 197–201°.

Phenylhydrazine derivatives of the new ester.

Phenylhydrazone. A solution (6 cc.) containing 100 mg. of the free ester was treated at room temperature with 0·2 cc. phenylhydrazine dissolved in acetic acid. A pale yellow crystalline precipitate formed within a few minutes;

[1] These melting-points were determined in the way described by Robison and King [1931, p. 329].

but the solution was allowed to remain at 0° overnight before filtration. The product was recrystallised twice from warm 75 % ethyl alcohol, from which it separated quickly at 0° in very pale yellow crystals, M.P. 144–145°.

Analysis. After being dried over sulphuric acid the substance did not lose weight in 1½ hours at 79° in the Pregl drier.

It gave P, 6·82 %.

Calculated for $C_{18}H_{27}O_8N_4P$, P, 6·78 %.

The phosphorus content corresponded with that of the phenylhydrazine salt of the phenylhydrazone of hexosemonophosphoric acid.

Phenylosazone. A solution (6 cc.) containing 100 mg. of the free ester was treated with 0·5 cc. phenylhydrazine dissolved in acetic acid, and heated in a boiling water-bath for 1 hour. After cooling to 0° the precipitate was filtered off and the solution again heated for 1 hour, cooled and filtered. The combined precipitates were recrystallised twice from boiling ethyl alcohol to which an equal volume of boiling chloroform was added, the solution being then left at 0° for 24 hours. It separated in fine yellow needles; M.P. 154–155°.

Analysis. The substance, dried over sulphuric acid, gave P, 5·69 %.

Calculated for $C_{24}H_{31}O_7N_6P$, P, 5·68 %.

The phosphorus content corresponded with that of the phenylhydrazine salt of the osazone of hexosemonophosphoric acid.

A specimen of osazone prepared from fructosediphosphate and similarly purified also melted at 154–155°, as did an intimate mixture of the two substances. It was, therefore, probable that this derivative of the new ester was identical with the osazone obtained from fructosediphosphate, *i.e.* the phenylhydrazine salt of the osazone of fructosemonophosphate (and of glucosemonophosphate).

The properties of the sugar obtained from the ester by acid hydrolysis confirmed the supposition that the compound was a monophosphoric ester of mannose; and the formation of a sparingly soluble phosphohydrazone also accorded well with this view. The identification of osazones by their melting-points is not, perhaps, conclusive; but such evidence pointed strongly to the identity of the osazone of the mannose ester with that obtained from hexosediphosphate. This would imply that the position of the phosphate group is the same in both compounds and that the new ester is probably mannose-6-phosphate.

Comparison of the rates of hydrolysis of mannosemonophosphate and glucosemonophosphate.

It has been shown that the hydrolysis of the new ester by 0·1 *N* H_2SO_4 proceeded at the same rate as that of glucosemonophosphate under similar conditions. The rates of hydrolysis of the two esters were further compared in the following experiments.

Hydrolysis by N *HCl at 100°.* The ester solutions were prepared by dissolving 85 mg. of the air-dry barium salts together with 40 mg. of K_2SO_4 in 20 cc. of

N HCl and centrifuging at once, to remove barium sulphate. Quantities of about 1·3 cc. of the solutions were introduced into a number of small, thin-walled, glass tubes, previously drawn out, which were then sealed up and dropped into a bath of vigorously boiling water. The bath was kept loosely covered so that the temperature was maintained at 100°. Two tubes were removed at each of the stated intervals and the inorganic phosphate was determined colorimetrically in 1 cc. or 0·5 cc. of each of the solutions. The total P was estimated in the original solution and again at the end of the hydrolysis. The concentration of the ester in each case was approximately 0·01 *M*.

The results, set out in Table III, show that the mannose ester was hydrolysed at a slightly faster rate than glucosemonophosphate and that this rate was maintained during the entire period in which 94·5 % of the phosphate was set free. The values of k are calculated for a unimolecular reaction,

$$k = \frac{1}{t_2 - t_1} \log \frac{a - x_1}{a - x_2},$$

t being the time in minutes.

Table III. *Hydrolysis in* N *HCl at 100°.*

	Glucosemonophosphoric acid (0·301 mg. P per cc.)		Mannosemonophosphoric acid (0·287 mg. P per cc.)	
Time hrs.	Hydrolysis %	$k \times 10^3$	Hydrolysis %	$k \times 10^3$
4	12·7	0·25	16·8	0·33
8	21·6	0·20	28·7	0·28
20	44·9	0·21	53·9	0·26
45	75·0	0·23	82·5	0·28
70	88·9	0·24	94·5	0·33

Autolysis of the free esters at 100°. Ester solutions of similar concentration (0·01 *M*) were prepared by dissolving the barium salts in water containing the exact equivalent of sulphuric acid and centrifuging. The method of heating was the same as that described above.

The results, given in Table IV, show that the hydrolysis of both esters occurred in these solutions at exactly the same rate, and, further, that this rate was nearly the same as that for the hydrolysis in *N* HCl. In the case of glucosemonophosphate the hydrolysis was actually more rapid in the ester solution containing no additional acid than in *N* HCl, which is in agreement

Table IV. *Autolysis in solutions of the free esters at 100°.*

	Glucosemono-phosphoric acid (0·285 mg. P per cc.)		Mannosemono-phosphoric acid (0·306 mg. P per cc.)		Fructosemono-phosphoric acid (0·294 mg. P per cc.)	
Time hrs.	Hydrolysis %	$k \times 10^3$	Hydrolysis %	$k \times 10^3$	Hydrolysis %	$k \times 10^3$
4	14·1	0·28	14·0	0·27	18·8	0·37
24	60·6	0·28	61·2	0·29	77·8	0·46
48	85·3	0·29	85·0	0·29	96·2	0·53

with the previous findings of Robison and King [1931]. The value of k for the autolysis of glucosemonophosphate is somewhat higher than that found previously for more concentrated solutions (0·03 M) by Robison and King, who observed that the value rose as the hydrolysis proceeded.

Isolation of fructosemonophosphate from the hexosemonophosphate of fermentation.

Fractional crystallisation of the brucine salts from further preparations of the fermentation hexosemonophosphate again yielded salts approximating in their properties to the Neuberg fructosemonophosphate. As these still contained a small proportion of aldose esters the barium salts were submitted to oxidation by bromine in the presence of baryta, followed by partial precipitation from their aqueous solutions by alcohol, to remove the sparingly soluble phosphohexonates.

The analytical values obtained for the purified salt agreed closely with those for the Neuberg fructosemonophosphate, the H. and J. reducing power being now within the limits found for the pure ester. It should be noted that somewhat variable values have been recorded for the specific rotation of the Neuberg ester, which is obtained by partial hydrolysis of hexosediphosphate; and this may possibly be accounted for by the presence of traces of the second hydrolysis product, which is strongly laevorotatory [Macleod and Robison, 1932].

Hydrolysis of fructosemonophosphate by acid.

The hydrolysis of the fructosemonophosphate isolated from the fermentation product was investigated under similar conditions to those employed for the aldose ester.

Hydrolysis in N *HCl at 100°.* The ester solution, of approximately 0·01 M concentration in N HCl was prepared, and the hydrolysis carried out in the manner described, except that the time intervals were much shorter. The results are set out in Table V and show that hydrolysis proceeded at a constant rate during 3 hours while over 83 % of the phosphate was liberated. The final value (92·5 %) gives a slightly lower rate; but it should be noted that the

Table V.

Time hrs.	Fructosemonophosphate (0·287 mg. P per cc.) Hydrolysis %	$k \times 10^3$	Lohmann's values for the Neuberg ester Hydrolysis %	$k \times 10^3$
0·5	24·4	4·05	20·1	3·23
1	45·3	4·68	36·9	3·23
1·5	59·6	4·39	—	—
2	70·3	4·45	62·5	3·76
3	83·6	4·30	75·0	2·93
5	92·5	2·83	84·9	1·81
8	—	—	87·4	0·44
13	—	—	90·4	0·39
33	—	—	93·0	0·14

possible errors in the P determinations have a very marked effect on the value of k when hydrolysis is approaching completion.

In the same table are given Lohmann's [1928] values for the hydrolysis of the Neuberg ester by N HCl under conditions similar to those here described. Lohmann, who applied this method to the quantitative examination of the hexosephosphates, concluded that the Neuberg monophosphate, like the parent hexosediphosphate, consisted of a mixture of esters; and his figures certainly justify this conclusion for the preparations which he examined. It is obvious that, if the hexosediphosphate contains any esters only slowly hydrolysable by acids, these would accumulate in the monophosphate fraction, obtained from it by partial hydrolysis, which represents the Neuberg ester. The question, therefore, resolves itself into the degree of purification of the hexosediphosphate or what shall be considered to constitute this fraction. Preparations of this ester, which have been very highly purified in this laboratory, show little indication, from their hydrolysis curves, of the presence of other compounds; but some evidence has emerged that other esters, forming barium salts of similar solubility to barium hexosediphosphate, may occur in the fermentation products.

It is clear from the rates of hydrolysis that the fructosemonophosphate, whose isolation from hexosemonophosphate is here described, contained a much higher proportion of the main constituent than Lohmann's ester. This is shown both by the higher initial value found for k and by the persistence of this value until a larger proportion of the total ester had been hydrolysed. It may be inferred that the substance contained less than 5 % of other esters[1].

Autolysis of the free ester at 100°. The rate of hydrolysis of the free fructosemonophosphoric acid in approximately 0·01 M solution containing no additional acid was also determined. The results, which are given in Table IV, show a quite different behaviour of this ester from that of the two aldosemonophosphates. The rate of hydrolysis in absence of HCl is, initially, less than one-tenth of the rate in N HCl, but increases somewhat as the hydrolysis proceeds. One must conclude that, in the case of the aldose esters, two essentially different compounds are being hydrolysed in the solutions of different acidity, while this is not so in the case of the fructose ester. The similarity in the values of k for the three esters, in 0·01 M solutions containing no additional acid, suggests that the compounds present in these solutions are of similar type, while, in presence of N HCl, the aldose esters pass over into another type, which is hydrolysed much less rapidly. The fructose ester cannot, or does not so readily, change into the second type and its rate of hydrolysis consequently increases with increasing hydrogen ion concentration.

Since the phosphate group is probably in the same position, 6, in the three esters, the formation of the stable 1:5 oxygen bridge is possible in glucose-

[1] Subsequent fractional precipitation of this product from aqueous solution by alcohol removed further traces of a sparingly soluble salt, probably phosphohexonate. The soluble portion was now hydrolysed by N HCl to the extent of 96·3 % in 5 hours and 98·3 % in 7 hours.

and mannose-monophosphates, but not in fructosemonophosphate. It is conceivable, therefore, that the easily hydrolysable forms present in the aqueous solutions of all three esters may represent either the furanose derivatives or possibly the free aldehydes or ketone, while the less readily hydrolysable compounds formed in N HCl or H_2SO_4 may be pyranose derivatives containing the stable oxygen ring.

It may be recalled that the phospho-osazone common to all three esters which contains no oxygen bridge is hydrolysed by N H_2SO_4 even more rapidly than fructosemonophosphate [Robison and King, 1931]. The investigation of the hydrolysis of these esters and their derivatives is being continued and may throw more light on their structure and on the causes of their reactivity.

Other facts which may be related to the existence of these dual forms are (*a*) the marked increase in the specific rotation of the glucose and mannose esters, especially the latter, on liberation from their salts, and the further increase shown in presence of 0·1N and N H_2SO_4, (*b*) the very low iodimetric reducing power of the mannose ester. This has not yet been satisfactorily accounted for. Slightly higher values than those shown in Table I were obtained by varying the conditions of the oxidation, particularly by increasing the concentration of potassium iodide in the iodine solution; but the highest figure corresponded with only 70 % of that theoretically demanded.

Other constituents of the fermentation hexosemonophosphate.

Some progress has been made towards the isolation of the fourth ester, which is responsible for the low H. and J. reducing power of the fermentation hexosemonophosphate, compared with that of the three known components.

This ester becomes concentrated in the final mother-liquors during the crystallisation of the brucine salts. These mother-liquors were evaporated to dryness, the residues recrystallised from methyl alcohol, the mother-liquors again evaporated and these operations repeated. A crystalline fraction, obtained by adding ether and light petroleum to some of these alcoholic mother-liquors, gave the barium salt No. 10, whose analysis is shown in Table I, while No. 11 was obtained from mother-liquors at a similar stage.

Attempts to purify the brucine salt of this ester by repeated crystallisation from various solvents gave products of higher reducing power. These salts are not yet homogeneous but certainly contain a high proportion of a new ester whose reducing power is very much lower than that of the three known hexosemonophosphates.

Fractional precipitation of the barium salt by alcohol or of the lead and basic lead salts gave products differing in little except the $[\alpha]_{5461}$, which was higher in the more soluble fractions. This may have been due to traces of trehalosephosphate.

Investigation by fractional acid hydrolysis, by bromine oxidation and other methods is being continued. One property common to these fractions may be mentioned. When heated with orcinol and hydrochloric acid a green colour is

developed, but the absorption band of the amyl alcohol solution is not identical with that given by pentoses, but is situated further towards the red end of the spectrum. The salts contain only traces of nitrogen as determined by a micro-Kjeldahl method.

Discussion.

The isolation of a mannosemonophosphate from the products of alcoholic fermentation fulfils an expectation long held in these laboratories. It was shown by Harden and Young [1909] that the fermentation of mannose as well as that of glucose and fructose by yeast juice responded to the addition of phosphate and that the same hexosediphosphate was formed from all these sugars [Young, 1909]. The deduction that at some stage in the esterification the three hexoses pass through a form which is common to them all leads to the further conclusion that, since monophosphoric esters of glucose and fructose are simultaneously formed from both these sugars, a corresponding ester of mannose should also occur among the products of their fermentation.

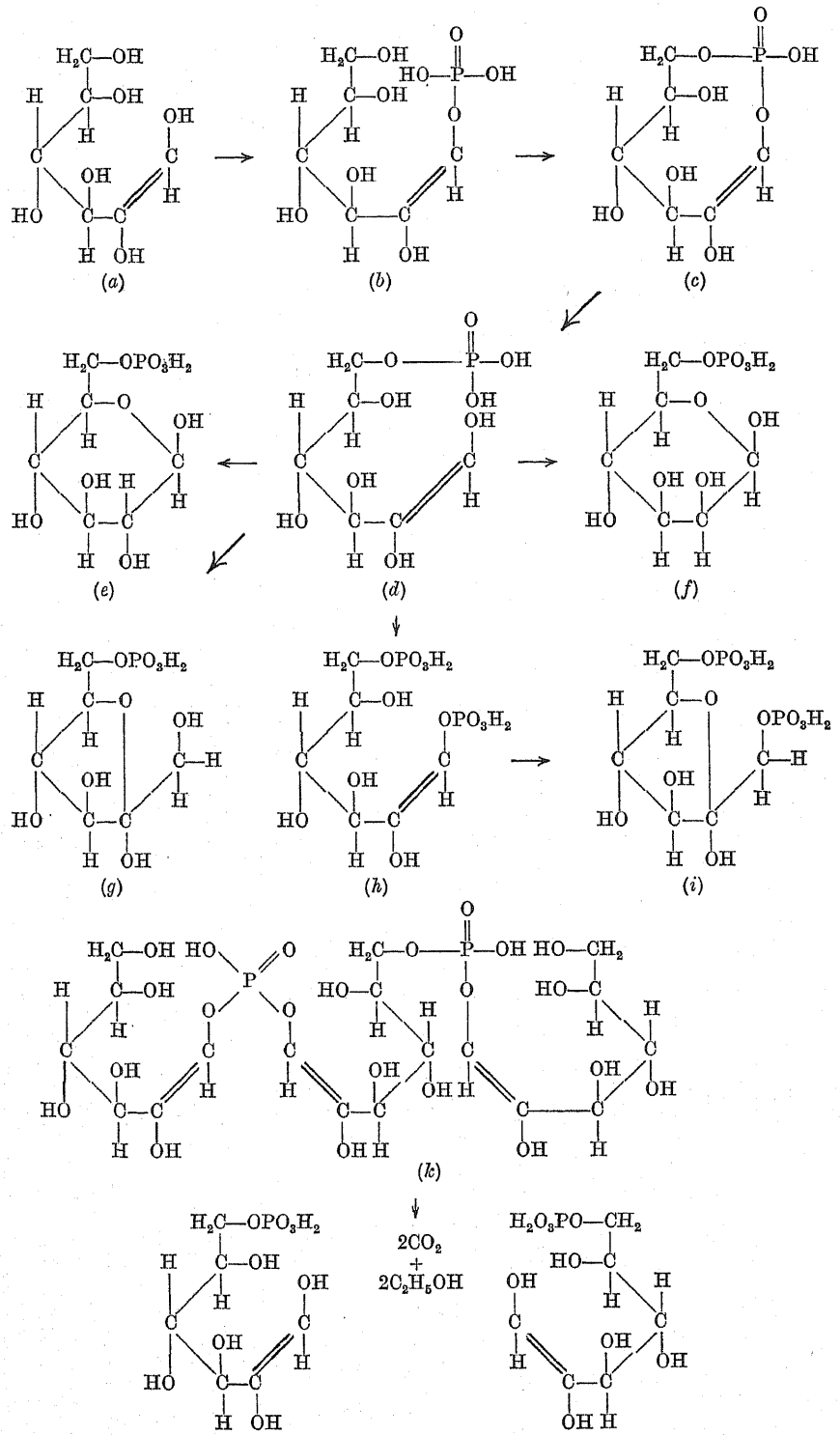
A scheme which has been suggested by the author [Robison, 1932] to account for the formation of these esters may be briefly recapitulated here. It is based on Armstrong's [1904] suggestion that the substance actually fermented by yeast is the enolic compound common to glucose, fructose and mannose. It is supposed that this enolic compound (*a*) (p. 2200) reacts rapidly with phosphoric acid, one molecule of which becomes attached to carbon atom 1 (*b*). This phosphoric group then wanders to carbon atom 6 *via* the intermediate compound (*c*) (*cf.* the wandering of the acetyl group in 1:2:3:4-tetra-acetyl-β-glucose under the influence of alkali [Fischer, 1920; Haworth, Hirst and Teece, 1930]). The resulting ester (*d*) by the addition of water to carbon atoms 1 and 2 would yield hydrated forms of glucose-, mannose-, and fructose-phosphates. Subsequent removal of water from the hydrated glucose and mannose esters could give 6-phosphoglucopyranose (*e*) and 6-phosphomannopyranose (*f*) while the hydrated fructose ester could yield only the γ-derivative, 6-phospho-fructofuranose (*g*).

The enolic group of ester (*d*) could, however, react with a further molecule of phosphoric acid, giving the diphosphoric ester (*h*) which would yield 1:6-diphosphofructofuranose (*i*), the hexosediphosphate of Harden and Young.

During the hydrolysis of these esters by phosphatases (of yeast or of bone) this series of changes may be supposed to take place in the reverse direction.

This scheme although it explains the formation of the phosphoric esters of fermentation does not account for the decomposition of half a molecule of hexose which accompanies the introduction of each phosphoric group during the fermentation.

It is conceivable that three molecules of hexose become linked together through two molecules of phosphoric acid and that the complex ester (*k*) so formed then breaks down, yielding two molecules each of hexosemonophosphate, carbon dioxide, and alcohol.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(k)
2CO2
+
2C2H5OH

Two molecules of monophosphate could similarly become linked with another molecule of hexose through two additional molecules of phosphoric acid, and this new complex ester, on disruption, would yield two molecules each of hexosediphosphate, carbon dioxide, and alcohol. The ratio

$$\frac{CO_2 \text{ produced}}{PO_4 \text{ esterified}}$$

would then be equal to unity for both stages of esterification.

We have, however, at present no evidence to prove that these assumptions are correct. The isolation and identification of the still unknown esters in the fermentation products may help us to answer this question.

The mannosemonophosphate described in this paper was obtained from the mixed products of fermentations of glucose and of fructose, so that further evidence is required to prove that it is formed from each of these sugars. The investigation of the ester was strictly limited by the small amount available and more work and more analyses are necessary to substantiate the conclusions arrived at. It is probable that the purest products were almost free from fructosemonophosphate. The absence of small amounts of glucosemonophosphate is more difficult to prove[1].

The purification of the ester by means of its phenylhydrazone is being attempted and should yield a homogeneous product. Fortunately, a much easier method has been found for the preparation of the ester than the very laborious fractionation hitherto carried out. Experiments by Dr Jephcott and the author on the fermentation of mannose by dried yeast in presence of phosphate have shown that at high temperatures (37°) esterification proceeds in a somewhat abnormal manner; a very large proportion of the total phosphate is converted into hexosemonophosphate which consists almost entirely of the mannose ester.

An account of these experiments will shortly be published and the investigation of the relationship between this abnormal esterification and production of CO_2 is being continued. It is apparent that such abnormal esterification would require some modification in the scheme outlined above.

The fermentation and esterification of mannose at 37° by fresh yeast in presence of toluene has been investigated by I. Neuberg and Ostendorf [1930], who also observed a greatly increased production of the monophosphate fraction compared with the amount formed from glucose. The products isolated by these authors, however, showed a high dextrorotation, $[\alpha]_D$, + 45° and + 34°, for the free acid, and a low P content (7·19 %). It may be suggested that this compound possibly contained mannosemonophosphate mixed with trehalosemonophosphate. The particular sample of dried yeast used by Jephcott and Robison produced much less trehalose from glucose and fructose than did those previously examined and this may have accounted, to some extent, for the purity of the mannose ester which they obtained.

[1] The synthesis of mannose-6-phosphate is at present being attempted by Mr V. N. Patwardhan and the author.

SUMMARY.

1. A third component has been isolated from the hexosemonophosphate produced by fermentation of hexoses with yeast juice. This compound has been shown to be a monophosphoric ester of mannose.

2. The $[\alpha]_{5461}^{20°}$ of the free ester is + 15·1°, and of the barium salt + 3·5°. The H. and J. reducing power of the ester is the same as that of glucose- and fructose-monophosphate. The iodimetric reducing power is only 60–70 % of the value theoretically required for an aldosemonophosphate.

3. The ester yields a sparingly soluble phenylhydrazine salt of a phenylhydrazone (M.P. 144–145°), and a phenylhydrazine salt of an osazone (M.P. 154–155°), which appears to be identical with that obtained from fructose-diphosphate and from glucose- and fructose-monophosphate.

4. From this it is inferred that the phosphate group is in the same position in all these esters, probably position 6.

5. The ester is very slowly hydrolysed by *N* HCl at 100° ($k = 0{\cdot}29 \times 10^{-3}$); the rate is slightly higher than that for glucosemonophosphate in *N* HCl at 100°, and is practically the same as the rate of hydrolysis of both the free esters in 0·01 *M* solution, containing no additional acid. The sugar produced by acid hydrolysis has the character of *d*-mannose.

6. The presence of the Neuberg fructosemonophosphate as a component of the fermentation hexosemonophosphate has been confirmed; and this ester has been isolated in purer form than that previously reported by Robison and King.

7. The hydrolysis of this fructosemonophosphate by acids at 100° has been examined. In *N* HCl the value of k was found to be $4{\cdot}36 \times 10^{-3}$, but in 0·01 *M* solution of the free ester the initial value of k was $0{\cdot}37 \times 10^{-3}$, only slightly higher than that of glucose- and mannose-monophosphate.

8. The bearing of these rates of hydrolysis on the structure of the three esters is discussed.

9. The reactions leading to the formation of fructosediphosphate and of the three monophosphates during the fermentation of hexoses are discussed, and a scheme is suggested to explain the facts at present known.

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CCLXI. THE USE OF PURE PHOSPHOTUNGSTIC ACIDS IN THE PRECIPITATION OF BASES.

I. METHOD OF CHECKING THE PURITY OF 1:24-PHOSPHOTUNGSTIC ACID.

II. THE INFLUENCE OF HYDRION CONCENTRATION AND SOME OTHER FACTORS ON THE PRECIPITATION OF BASES.

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Introduction.

With the exception of fractionation of phosphotungstates of bases by acetone-water mixture, no attempts seem to have been made to modify the use of phosphotungstic acid [Drechsel, 1887]. It has been shown by one of us [Peters, 1930] that some bases are characterised by the hydrion concentration at which precipitates appear with neutralised phosphotungstic acid (sodium phosphotungstate). Extended use was made of these observations in concentrating vitamin B_1 by Kinnersley and Peters [1930]. As the methods there employed seemed likely to have general application, a further study was undertaken.

The primary object of our work was practical, but it was hoped also to clear the ground for theory as to the conditions governing precipitation and composition of the precipitates. Much has been learnt, but it is clear that the work only constitutes a preliminary survey of a complicated field. Fundamental observations upon the separation of complexes at various c_H are being made by Britton and his colleagues [1932], to which this problem is clearly related. Previously, the purest commercial acid had been used. Here we have concentrated mainly upon the 1 : 24-phosphotungstic acid, though some observations have been made with the 1 : 18-acid. The results are not the same as those with the commercial acids.

PART I. METHOD OF CHECKING THE PURITY OF 1:24-PHOSPHOTUNGSTIC ACID (By H. BARNES).

Phosphotungstic acid, even of the best commercial grades, is a mixture, usually considered to approximate to 10 % of the 1 : 18-acid and 90 % of the 1 : 24-acid. Throughout, the ratios expressed refer to the ratio $P_2O_5 : WO_3$.

Among the numerous acids described in the literature [Scheibler, 1872; Sprenger, 1880; Kehrmann, 1887; Kehrmann and Fraenkel, 1891; Brandhorst and Kraut, 1888; Gibbs, 1877; Péchard, 1889], 1 : 12, 1 : 16, 1 : 21, 1 : 22, 1 : 18 and 1 : 24, the last two are the most clearly established. They have been prepared for the purpose of this work by the method of Wu [1920] from Kahlbaum's highest grade of sodium tungstate. A less reliable sample upon one occasion gave a 1 : 24-acid of green-yellow tint, possibly owing to the presence of sodium molybdate [see Folin and Marenzi, 1929]. The final product was best recrystallised from a small amount of water allowed to evaporate over sulphuric acid in a desiccator at room temperature, giving large octahedra. The usual yield of the purest acid from 200 g. of tungstate was 20 g. of the 1 : 24-acid. The yield was improved upon one occasion by cooling all the reagents thoroughly at the ether extraction stage. It was found important to store the crystals in a closely stoppered bottle to prevent the efflorescence which easily alters their composition. Even with considerable precautions the original analysis figures were not given after storing for a few weeks.

Only one form of the 1 : 18-acid was used in this work, obtained from an ammonium salt like Wu's B form by ether extraction, and consisting of pale greenish-yellow thick rhombohedral plates.

Analysis of the samples used.

In order to check the purity of the 1 : 24-acid used, a new method was devised, after unsuccessful work with other analytical methods. A short description of the experiments best indicates the difficulties and may be helpful to others. No success was obtained by the application of colorimetric phosphate methods to mixtures of sodium tungstate and potassium phosphate.

The following stages can be recognised in most previous work.

(*a*) The decomposition of the complex acid. Different investigators have used widely varying reagents from 20 % boiling sodium hydroxide for 30 minutes to dilute hydrochloric acid. Evidence is scanty for the efficacy of any particular method. Experiments performed during the progress of this research suggest that different acids require different treatment for disintegration.

(*b*) Precipitation (i) of the two acids together as a mixture of mercurous tungstate and mercurous phosphate; (ii) of the phosphate as magnesium pyrophosphate; (iii) of the tungstate as tungstic oxide, or as a compound with (iv) tannic acid or (v) benzidine.

Gibbs's method [1880][1]. After decomposition the tungstic and phosphoric acids were precipitated together in boiling acid solution with mercurous nitrate, as a mixture of mercurous tungstate and mercurous phosphate, the excess acid was neutralised with mercuric oxide, and the needles so obtained after washing with dilute mercurous nitrate solution and igniting gave the sum of P_2O_5 and WO_3. Gibbs states that no phosphorus was lost during the ignition, but von Knorre [1908] considers that some phosphorus was left in the filtrate from the mercury precipitation. On a second portion Gibbs determined the phosphate as $Mg_2P_2O_7$, after precipitation as magnesium ammonium phosphate. This latter method was worked out for Gibbs by Gooch [1879] and it is clear from his results that the separation of phosphoric and tungstic acids in this way is a matter of considerable difficulty. The phosphate always seems to be in error by + 6 to 8 % due to the adsorption of sodium tungstate from the solution on to the precipitate. This error is only slightly reduced, by taking up the magnesium ammonium phosphate precipitate in hydrochloric acid, and reprecipitating with magnesia mixture. Gibbs determined the WO_3 by difference.

[1] The earlier worker [Scheibler, 1872], has not given a method of analysis.

The rather unsatisfactory nature of these methods was shown by a re-investigation of the effect of sodium tungstate on the estimation of potassium hydrogen phosphate (Sørensen), as $Mg_2P_2O_7$, after precipitation as magnesium ammonium phosphate. The original work of Gooch was amply confirmed—the error in the first precipitation always being of the order of 10 to 20 %. Several reprecipitations were then tried, and only after four such reprecipitations of the magnesium ammonium phosphate could the error be reduced to approximately 1 %. In the experimental procedure adopted, the mixtures of sodium tungstate and potassium hydrogen phosphate were made up in amounts approximately equivalent to a 1 : 20-phosphotungstic acid, the solution was heated to boiling and magnesia mixture cautiously added with constant stirring. After cooling an excess of ammonia was added, and the mixture allowed to stand at least 4 hours before filtration. Table I gives the results.

Table I.

Amount of $Na_2WO_4, 2H_2O$ taken g.	Amount of KH_2PO_4 taken g.	No. of precipitations	$Mg_2P_2O_7$ found g.	KH_2PO_4 corresponding to $Mg_2P_2O_7$ found g.	Errors %
1·5641	0·0902	1	0·0876	0·1071	18·7
1·7697	0·1099	4	0·0912	0·1114	1·4
1·0718	0·1099	4	0·0914	0·1117	1·6

Naturally such a method is very tedious and subject to considerable manipulative error.

Other methods. Kehrmann [1887], after boiling the complex acid for half an hour with sodium hydroxide, precipitated the phosphate as magnesium ammonium phosphate, using magnesium and ammonium nitrates instead of the corresponding chlorides and, after evaporating down the ammoniacal filtrates, precipitated the tungstic oxide from them as such by addition of hydrochloric acid. Sprenger [1880] proceeded in a similar manner, but used tannic acid to precipitate the tungstic oxide.

A critical survey of the literature with some experimental work was published by von Knorre [1908]. He considered the method of Kehrmann for splitting up the complex acid, namely boiling with sodium hydroxide for half an hour, as far too vigorous, and he himself appears merely to have added hydrochloric acid. He also confirmed Gooch's experience on the difficulty of obtaining a pure magnesium ammonium phosphate. He states without giving experimental details that the determination of tungsten as oxide is not accurate in the presence of magnesium salts. With regard to the tannic acid method of Sprenger, von Knorre's experiments show that, using pure solutions of sodium tungstate, the results are always too low. In addition the exact conditions for a good precipitation by this method are difficult to obtain—the appearance of colloidal solutions being frequent. Von Knorre himself precipitates the tungstate first as benzidine tungstate in acid solution, adding a little sulphuric acid to help coagulation of the precipitate. On ignition the

benzidine tungstate gives tungstic oxide, and benzidine sulphate is volatilised. Phosphate is determined in the tungsten-free filtrate, as $Mg_2P_2O_7$. This method is open to several criticisms. First, the benzidine tungstate must be precipitated in acid solution, and the presence of acid will tend to re-form the complex acid from the sodium phosphate and tungstate, these being partly split again upon making alkaline for the phosphate precipitation; secondly, as von Knorre's experiments show, a small amount of phosphate is precipitated along with the benzidine tungstate, making reprecipitation necessary to free it completely from phosphate.

Wu's method [1920]. The essential stages for estimation of P_2O_5 are hot sodium hydroxide, precipitation as magnesium ammonium phosphate, ignition, digestion with HCl and reprecipitation of phosphate; for WO_3, ignition and determination of P_2O_5 in residue. Since P_2O_5 was found to be partly volatile, it is not clear why no P_2O_5 is lost in the first process.

This method was tried with the exception that the precipitations of magnesium ammonium phosphate were made in hot solutions. Table II shows some results with control mixtures of phosphate and tungstate[1]. The amount of phosphate (calculated as $Mg_2P_2O_7$) after subtraction of adherent sodium tungstate was not equal to the phosphate originally taken. After the digestion stage, the amount recovered however was in good agreement.

The incomplete removal of tungstic oxide by the digesting process in our hands is shown in the first experiment in Table II and a complete phosphate analysis according to Wu is shown in the second experiment of the same table.

Table II.

Na_2WO_4, $2H_2O$ g.	KH_2PO_4 g.	$Mg_2P_2O_7$ on 1st precipitation g.	Error in 1st precipitation %	WO_3 remaining after HCl g.	Corrected error %	$Mg_2P_2O_7$ on 2nd precipitation g.	Error in 2nd precipitation %
1·5641	0·0902	0·0876	18·7	0·0015	16·8	—	—
0·7833	0·1315	0·1214	12·9	0·0033	8·9	0·1067	0·8

The methods of separation of these mixtures proved so tedious and uncertain that a trial was made of ignition.

The ignition method. It has been stated [Rosenheim and Jaenicke, 1917] that upon heating to a dull red heat, these phosphotungstic acids give off most of their water, leaving behind only metaphosphoric acid and tungstic oxide. The latter should not volatilise until about 1400°, whereas the former should be volatilised by about 800°. By heating the phosphotungstic acid first at 540° to constant weight and then at 1000°, for the best samples practically theoretical figures could be obtained for water and tungstic oxide.

The acid was heated in a crucible supported on a small silica stand in an electric furnace; the temperature was regulated by a variable resistance and was read off directly on a calibrated thermocouple. Heating for some days at

[1] Wu did not publish any figures for controls.

540° and 1000° to drive off water and phosphorus compounds respectively was required. The process however needs little attention. Table III shows the results. There is some uncertainty as to the exact temperature of volatilisation of metaphosphoric acid, and it seems to depend to some extent upon conditions. We have therefore calculated the results expected in two ways, giving respectively the theoretical figures upon assumption that (*a*) the whole of the water disappears at 540°, leaving behind P_2O_5, and that (*b*) one molecule remains, forming metaphosphoric acid.

Table III.

Acid taken g.	H_2O lost at 540° g.	H_2O %	WO_3 left at 1000° g.	WO_3 %
2·3431	0·3864	16·48	2·0995	81·54
1·2880	0·2101	16·31	—	—
3·6913	0·6071	16·45	2·9908	81·02
2·1354	0·3466	16·24	1·7342	81·21
	Average	16·37		81·38

Formula for 1:24-acid: $3H_2O.P_2O_5.24WO_3.60H_2O$.
(*a*), (*b*) Calculated for $60H_2O + 3H_2O$ on assumption (*a*) that all H_2O volatilises at 540° and (*b*) that 1 molecule H_2O remains with P_2O_5.

		Calculated	
	Observed	(*a*)	(*b*)
%	%	%	%
H_2O	16·37	16·56	16·31
WO_3	81·38	81·40	81·34

The figures are so close to the theoretical that there can be little doubt that the method is of value for the 1 : 24-acid. Curiously enough it proved impossible to control it by heating mixtures of sodium tungstate and phosphate; the conditions of volatilisation seemed to be altered. One experiment upon the 1 : 18-acid suggested that it might be also of value for this acid, but it seemed to take longer to attain constant weight.

Conclusion.

The purity of 1 : 24-phosphotungstic acid can be checked by heating successively to constant weight at 540° and 1000°.

PART II. THE INFLUENCE OF HYDRION CONCENTRATION AND SOME OTHER FACTORS ON THE PRECIPITATION OF BASES

(By H. BARNES and R. A. PETERS).

We have studied the influence of concentration of base and of phosphotungstate together with salts upon the initial precipitation. Most work has been done with creatinine, rather less with histidine, and least with a few other bases. More attention has been paid to the 1 : 24-acid, whose greater stability makes it a more useful reagent.

Methods.

Initial point of precipitation. An aqueous solution of the base (1·0 cc.) was treated with 0·1 cc. Na phosphotungstate solution (p_H 6·0). Acid (usually sulphuric) of convenient strength was added from a graduated pipette until a faint permanent cloud was observed. Observation of the initial point was aided by the use of a point source of light in a dark room, using a comparison tube. The sharpness of this point varied with the substance, being very definite with histidine. With creatinine it was much influenced by the speed of addition of the acid (see below).

p_H *range of precipitation.* The base was dissolved in 5·0 cc. water and divided into 1·0 cc. samples (Ostwald pipette). After adding 0·1 cc. of phosphotungstate (90 %) to each, and the requisite sulphuric acid of appropriate strength, the tubes were centrifuged after standing for half an hour. The liquid was used for the determination of p_H and the residue washed, dried and weighed.

Hydrion concentration. As the hydrogen and quinhydrone electrodes were inapplicable with the unstable phosphotungstates, observations were made colorimetrically, or where necessary with the glass electrode. In the latter case duplicate observations agreed to within 0·03 p_H.

Chemical substances.

Creatinine. Commercial samples were purified (1) by way of the zinc chloride compound and (2) by repeated purification from acetone [Edgar and Hinegardner, 1923]. A theoretical value was obtained for nitrogen.

Histidine. More than one sample was used, some from commercial sources, and others prepared in the laboratory by the methods of Raistrick [see Cole, 1926], and of Vickery and Leavenworth [1928]. The melting-points of the compounds varied, but no essential difference was found in the precipitation behaviour. A pure specimen of the dihydrochloride melting at 242° gave the same results as others.

Guanidine sulphate. Recrystallised from commercial sources.

Aminoguanidine sulphate, M.P. 207°. The specimens of *as*-dimethylguanidine and of arginine nitrate were from commercial sources and of doubtful purity.

Phosphotungstate solutions.

An aqueous solution of the phosphotungstic acid was neutralised with NaOH to p_H 6·0 approx., usually in presence of a trace of bromocresol purple. For weaker concentrations 10 % NaOH is sufficient. For the 90 % solutions, which are nearly saturated, it is best to dissolve in the minimum amount of water and add slowly 20 % NaOH cooling the solution between each addition. The use of stronger NaOH or taking to p_H 7·5, or allowing the solution to get hot is apt to cause decomposition. The weaker solutions appeared to keep indefinitely at room temperature, but the stronger ones often deposited crystals after standing. It is not known whether these have a different composition.

Creatinine.

This base was chosen because it was relatively easy to obtain, and had an initial precipitation point lying between p_H 5·0 and 6·0. This point was investigated with amounts of phosphotungstate (*a*) much less than and (*b*) more than were required to precipitate amounts of dissociated creatinine up to 8·0 mg. per cc. (0·0707 *M*). For (*a*) 0·1 cc. of 10 % phosphotungstate was added to 1·0 cc. solution containing the base, and for (*b*) 0·1 cc. of 90 %. As the amounts of acid needed to start precipitation varied, the concentra-

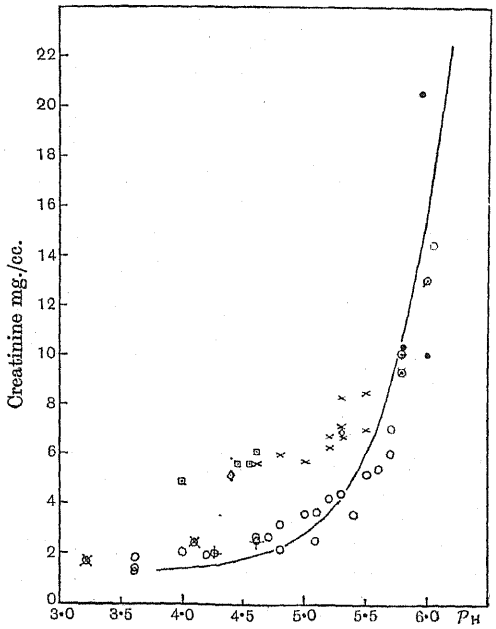


Fig. 1. Variation of initial point of precipitation of creatinine with concentration. Concentrated phosphotungstate solution (1:24). Ordinates creatinine concentration mg./cc., 11·3 mg./cc. = 0·1 *M*. Abscissae p_H.

⊙ × Points at 20°. ◈ 10°. ⊡ 31°. ⊠ Na_2SO_4 added. ⊕ NaCl added.

tions of phosphotungstate at the moment of precipitation might be as an extreme 4·5–8·0 %, and one-tenth of this respectively. With (*b*) points above 7–8 mg./per cc. creatinine, were well defined and could be obtained to within 0·1 p_H. Below this the conditions of precipitation became very important. The lowest points in Fig. 1 (circles) were obtained by one observer working to standard conditions, and represent an extreme. Other points (crosses) are observations of others. When the acid is added very slowly, there is no question that the initial point tends to be pushed to the acid side. The area containing the points is really an indeterminate zone. In (*a*) (Fig. 2) the same phenomena are to be seen. The weaker concentration of phosphotungstate is associated

with a shift of approximately 0·6 p_H for 10–20 mg. creatinine concentration. A few points are included with the 1 : 18-acid.

The results with the 10 % phosphotungstate can be compared directly with those previously published for the commercial acid [Peters, 1930] and are seen to be considerably further to the alkaline side. Commercial acid p_H 2·5, 2·0 mg./cc.; p_H 4·0, 10 mg./cc., and for 1 : 24-acid p_H 4·0 and 5·8; for 1 : 18-acid the values are p_H 5·2 and 6·2. With the 1 : 24-acid above 6·0 mg./cc. creatinine, the initial point becomes largely independent of concentration of base. We have included in the diagram points found in the presence of added sodium chloride and sulphate up to 2·5 % concentration

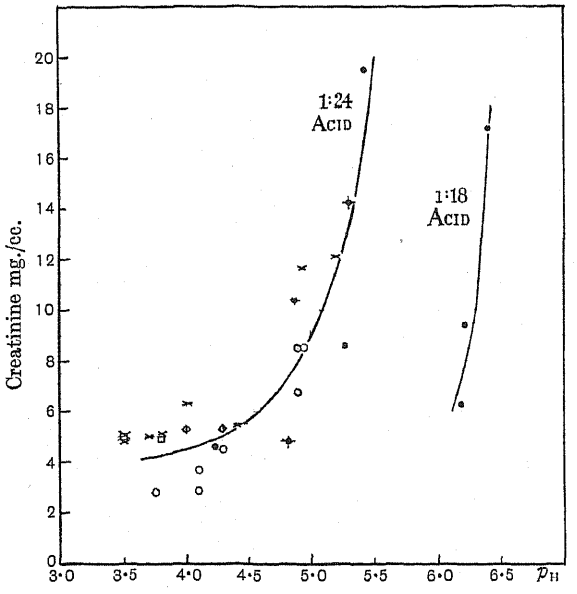


Fig. 2. Variation of initial point of precipitation of creatinine with concentration, for 1:24-phosphotungstate (dilute) and 1:18-phosphotungstate (dilute 10 %). Data as Fig. 1.

and also variations of temperature of $\pm$ 10°. The differences so introduced were slight if at all existent, and were negligible so far as these experiments were concerned. Nor did it make a significant difference if HCl were substituted for H_2SO_4. That traces of creatine could not influence results was also shown upon two occasions by adding creatine; this effectually controlled the possibility of traces of creatine in the creatinine or formed in solution.

Estimation of the p_H range of precipitation showed that with a concentration of 5 mg./cc. base most of the precipitate appeared upon titrating from p_H 6·0 to 2·8; hence the practical issue is that if we collect all the phosphotungstate precipitated between p_H 6·0 and 2·5, we shall have all the creatinine except that which is soluble, some 1·6 mg./cc. or rather more depending upon the concentration of phosphotungstate used.

From the theoretical standpoint, the important general points seem to be (1) the constancy of the initial precipitation point at about p_H 6·1 for both acids when sufficient phosphotungstate is present, (2) the fact that the zone of precipitation extends from p_H 6·0 to 2·0 approximately, over 4·0 units of p_H. The special points are (3) the tendency for the initial precipitation point to shift to the acid side in the case of the 1 : 24-acid with decreasing concentration of phosphotungstate, and (4) the tendency for precipitation to take place more definitely near the initial point with the 1 : 18-salt. Dealing first with the general points, the p_K for creatinine has been shown by Cannan and Shore [1928] to be 4·91 at 15°. The precipitation is therefore roughly co-extensive with the range of dissociation of creatinine. This suggests that it is related to this dissociation. Now the complex precipitated might be either a salt-like compound with phosphotungstate, or some complex with the phosphotungstate molecule as a whole. We could exclude the formation of a salt-like compound upon passing from an acid to an alkaline p_H, if it happened that the dissociation of the phosphotungstic acid was complete at p_H 2·0. It would then be clear that a precipitate formed at p_H 5·0 must consist of a complex of Na phosphotungstate together with dissociated creatinine. In the absence of data in the literature, the dissociation curve of the complex acids has been briefly investigated to settle this point.

Dissociation curves of the phosphotungstic acids. The dissociation curve for the 1 : 24-acid as determined by titration is given in Fig. 3. Up to p_H 7·0 some 19 g.-mol. Na are taken up per 1·0 g.-mol. of the acid and at approximately p_H 7·8 24 g.-mol. Na, or an amount of Na corresponding to each unit of WO_3. It is curious and suggestive that the sudden change in the character of the dissociation curve starts at p_H 7·0 and becomes intense at p_H 7·8, when 24 acidic groups have been neutralised. The points are here apt to become uncertain, and there is evidence of change of equilibrium, because some time must be allowed to elapse after addition of alkali before the final p_H is reached; immediately after titration the p_H is much more alkaline than the equilibrium point. Further investigation is needed, but the above is sufficient to show that the phosphotungstic acid molecule must be regarded as a polyacidic micelle; the dissociation curves of the different acidic centres overlap to form an almost continuous straight line over the range p_H 2·0–7·0. The behaviour is rather analogous to that of a protein, though uncomplicated by basic dissociations. Since gradual neutralisation of the acid occurs over the whole range p_H 2·0–7·0, we cannot exclude the possibility that salt-like compounds between dissociated phosphotungstic acid and base contribute to the precipitate formation. Two kinds of compounds can be pictured, if we assume that no actual change is taking place in the phosphotungstate molecule itself, (1) some molecules form creatinine phosphotungstates, while others remain uncombined; (2) each phosphotungstate molecule acts as a polyacidic micelle, and takes up a certain proportion of base. Though the data do not admit of decision, we think that (2) is more likely. This would give precipitates, con-

sisting of Na-creatinine-phosphotungstate with some undissociated groups in the molecule according to the p_H.

The dissociation curve for the 1 : 18-acid was briefly investigated and proved to be different. Our supplies were limited and did not admit of detailed study, but there seemed to be no buffering action between p_H 2·0 and 7·0. The difference provides one avenue to explore in explaining the variations in precipitation behaviour between the two acids.

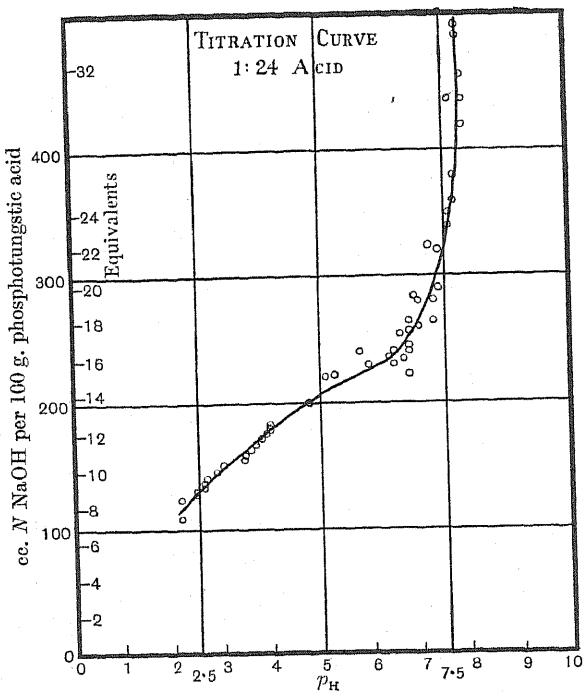


Fig. 3. Titration curve 1:24-acid. M equivalents NaOH.

Diagrammatically we should have

Phosphotungstic acid—undissociated groups (n)
Phosphotungstic acid—organic base (creatinine) (m)
Phosphotungstic acid—Na (p)

Here n, m, p represent whole numbers, the values of which vary according to the p_H and the concentration of phosphotungstate and Na.

Composition of the precipitates.

We have tested whether the precipitates contain sodium and found this present. They are clearly different from those formed by bases in acid solution. For instance according to Drummond [1918] 1 g. of creatinine gives in acid solution less than 10 g. phosphotungstate. In our case, 1 g. often gave 12–13 g. Experiments upon the relative proportion of Na and creatinine have been

made as follows. Approximately 100 mg. samples of creatinine have been treated with 1·5 cc. H_2O, 1·5–2·0 cc. 90 % Na phosphotungstate (p_H 6·0) added, and the p_H adjusted to 5·0 and 3·0 approximately. After standing for one hour or more, the precipitates were removed by centrifuge and the precipitate washed once with 1·0 cc. H_2O containing a trace of phosphotungstate at the p_H used. If taken within 1 hour, precipitation has not reached completion. They were not dried, owing to the possibility of introducing changes in composition and variations in the usual practical procedure. They were extracted as in the vitamin B_1 work by grinding with baryta and H_2O 4 times. The resulting solution was acidified with H_2SO_4 to Congo red, heated and made up to a known volume after filtration. One-fifth was taken for the estimation of creatinine by Folin's method, and the remainder evaporated to dryness and incinerated; the resulting Na_2SO_4 was determined by drying to constant weight in a crucible. Controls, in which known amounts of NaOH and creatinine were added to phosphotungstate at a given p_H and the whole was extracted with baryta, gave a recovery of 95–98 % for creatinine but not more than 75 % for sodium.

The experiments, some 10 in all, showed that Na was always found to be present in the baryta extracts, but the ratio creatinine/Na varied rather widely from the extremes of 10/1 to 4/1. The proportion of Na in the precipitates appeared to decrease when the precipitates were allowed to stand for periods over 1 hour. The fact that the weight of precipitate is some 12–13 times that of the creatinine taken is most simply explained upon the view that the precipitates contain on the average 5 molecules creatinine per molecule of phosphotungstic acid. Upon the assumption that creatinine is here monobasic, one molecule attached at one acidic grouping, using data from the dissociation curve, we may calculate that approximately at p_H 3·0 the ratio of creatinine/Na should be 4·7 and 3·4 at p_H 5·0. Incomplete recovery of Na will not explain the differences from the observed ratios, so that we are faced with the conclusion that upon balance each creatinine molecule displaces more than one Na. At p_H 5·0 replacement of 2Na would lead to a ratio of 12/1 and at p_H 3·0 a higher ratio still. The increase in the proportion of creatinine/Na upon standing must be due either to a continued replacement of Na in the precipitate by creatinine (a kind of base exchange like that of Ca in fuller's earth [compare Phelps, 1931]), or to the formation of phosphotungstates of more than one kind. In several cases crystals formed, so that the compounds must be well defined. Their evident complexity makes caution advisable in framing hypotheses.

If however the precipitation is due to dissociated creatinine, as seems most probable, the amount dissociated at a given p_H is known from the usual relation[1] $p_k + \log \frac{\alpha}{1-\alpha} = p_H$, where $p_k = 4{\cdot}91$. We can calculate for any p_H the total concentration needed to give an amount of dissociated creatinine equal

[1] p_k, apparent dissociation constant; α = ratio $\frac{\text{dissociated acid}}{\text{total acid}}$.

to the known solubility; upon hypothesis this should be proportional to the amount which just forms a precipitate. The curve in Fig. 1 is so calculated for a solubility of dissociated creatinine phosphotungstate of 1·25 mg./cc. The agreement with the points determined is sufficiently close to suggest that the fundamental assumption may be justified. In Fig. 2, a similar curve is drawn for a solubility of 4 mg./cc. In the latter case, there is a suggestion of discontinuity in the determined points, which was supported by the results of an attempt to determine the solubility product relations for low dilutions of phosphotungstate.

The concentration of phosphotungstic acid required to initiate precipitation at a constant p_H.

1·0 cc. solution (Ostwald pipette), containing 20 mg. dry creatinine, was treated with 0·264 cc. *N*/3 HCl unless otherwise stated to bring to p_H 4·90 approx. To this was added from a micro-burette, 4·95 % Na phosphotungstate solution. The p_H was then determined colorimetrically using a comparator. All solutions were stored in bottles protected from air with soda lime tubes to eliminate CO_2 as much as possible. Na phosphotungstate p_H 4·90 was made by neutralising 10 % 1 : 24-phosphotungstic acid in the ratio of 1·02 cc. 0·2 *N* NaOH to 1·0 cc. acid. Table IV A gives some results for immediate precipitation and IV B for precipitation upon standing for 24 hours.

Table IV. *Precipitation of creatinine by sodium phosphotungstate at constant* p_H.

In all cases 1·0 cc. creatinine solution (20 mg. creatinine) used.

0·264 cc. *N*/3 HCl added in Exps. 1, 2, 3, 8, 9, 10.
0·15 cc. ,, Exps. 4, 6, 7.
0·08 cc. ,, Exp. 11.
0·14 cc. ,, Exp. 12.

Exps. 11, 12 with 90 % Na phosphotungstate, the remainder with 4·95 %.

Exp.	cc. phospho-tungstate	p_H	Conc./cc. Crea-tinine (C)	Conc./cc. Phospho-tungstate (P)	C × P	
A. Temp. 18·5±0·5°. Immediate precipitation.						
1	0·605	4·8	10·7	15·6	167	
2	0·385	5·0	12·15	11·6	140	
3	0·300	4·9	12·8	9·5	122	
4	0·144	5·3	15·5	5·51	85	
5	0·150	5·3	15·4	5·71	85	
B. Temp. 25·5±0·5°. Slow precipitation or no precipitate (22 hours).						
6	0·02	5·3	17·1	0·085	1·45	Precipitation
7	0·01	5·3	17·2	0·043	0·7	Very small precipitation
8	0·05	4·9	15·3	0·189	2·9	Precipitation
9	0·035	4·9	15·3	0·133	2·04	Very small precipitation
10	0·02	4·9	15·3	0·077	1·2	No precipitation
11	0·10	6·0	17·0	70·6	1200	No precipitation
12	0·10	5·6	16·1	72·5	1160	Very small precipitation

For immediate precipitation, we have for p_H 4·8–5·0 a product of creatinine and phosphotungstate of 122–167 mg./cc., but at p_H 5·3 a product of 85,

i.e. less phosphotungstate seems to be required to produce an immediate precipitate at p_H 5·3. This suggests that the precipitates are complex, but no emphasis can be placed upon these figures; they are not true equilibrium points, because, upon standing for 24 hours, the precipitation tends to progress and beautiful crystals appear, of at least two different forms, needles and cubic prisms. These merit further study. Provided that the p_H is within the precipitation zone, surprisingly little phosphotungstic acid will suffice to give crystals in 24 hours, amounts far below those giving visible precipitates even within an hour (see Table IV B). It should be emphasised that outside the indeterminate zone, phosphotungstate and creatinine may be kept together in solution almost indefinitely without crystallisation or precipitation (see especially Exp. 11).

As a working hypothesis, it is suggested that the main precipitation is related to the presence of dissociated creatinine. The complete theory will have to take into account as well as the p_H the concentrations of creatinine and phosphotungstate, and probably the equilibrium between more than one form of compound.

Note. There is one possibility, which requires mention. It has been recently shown that at a liquid interface, there is an apparent shift of dissociation constant [Peters, 1931]; similar conclusions follow from work on charcoal adsorption for the solid interface [Phelps and Peters, 1929; Phelps, 1931]. This is likely to happen also with these precipitates, and probably provides a partial explanation of the change in character upon standing.

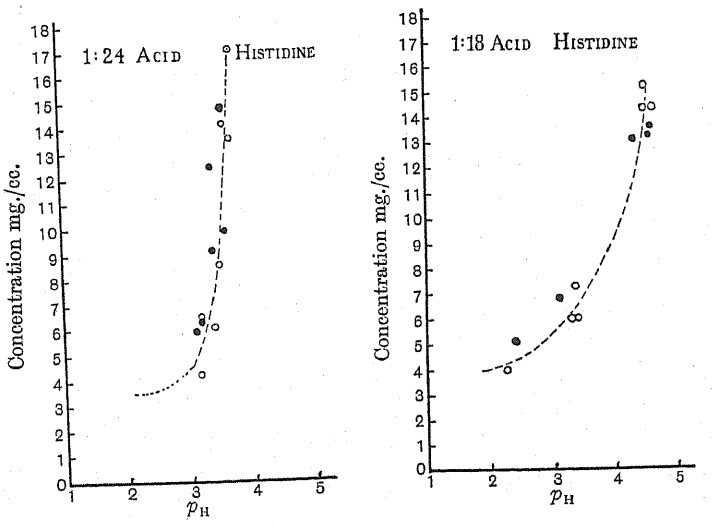


Fig. 4. Variation of initial point of precipitation of histidine (hydrochloride) with concentration. 1:24- and 1:18-acids. Concentration mg./cc., 9·1 mg./cc. = 0·1 *M*.
○ = 10 % acid. ● = 90 % acid.

Histidine.

The curves shown in Fig. 4 have been obtained by the same technique as in the case of creatinine, histidine hydrochloride being used. The curves for the 1 : 24- and 1 : 18-acids have the same position, differing in this from

creatinine. They bear the same relation to the dissociation curve p_H 1·0–4·0 (one of the dissociating groups of histidine) which we find in creatinine, but the same explanation does not apply. There is now much evidence for belief in the zwitterion constitution of the amino-acids [Bjerrum, 1923; Birch and Harris, 1930; Borsook and Wasteneys, 1930]. According to this view the group dissociating over this range is the —COOH grouping. Hence, we cannot take the simple explanation adopted for creatinine; we must believe that for histidine a precipitation of the compound formed over the range of dissociation of the —NH group is inhibited as long as the —COOH group is ionised. The matter could be tested by conductivity determinations. We should of course get precisely the same curve, but the initial point of precipitation would coincide with the dissociation of the —COOH group. This might explain the coincidence of the precipitation curves for the two different acids, because the compounds would already exist in solution at the p_H at which precipitation started.

Other bases.

We have not explored the field of the relation of chemical constitution to p_H of precipitation at all fully, and give the incomplete data in the belief that they may be of value to other workers. Fig. 5 represents the most probable value of the main precipitation point. Some considerable difference is made in some cases by the method of addition. For instance, for aminoguanidine sulphate, a very pure specimen, addition of Na phosphotungstate at p_H 5·0 to a solution containing 11 mg./cc. caused an opalescence. This became definite precipitation at p_H 7·4. The data should be studied in relation to those for the commercial acid. The points for this are seen to lie often intermediately between the very different points given by the 1 : 18- and 1 : 24-acids. With guanidine, as previously indicated, precipitation occurs on the alkaline side in low concentrations for the commercial acid and for the 1 : 24-acid, of which the commercial largely consists. It is more sharply defined for the 1 : 24-acid. Introduction of the amino-group modifies the precipitation, shifting the points to the alkaline side at a low concentration.

This alkaline precipitation by guanidine introduces the last complication which we have met, and seems to need another explanation, as it is related to the dissociation in the opposite sense. If $p_{K'_b}$ for guanidine is 8·5, then this compound only precipitates in low concentration when undissociated, and must be forming a complex. It is probably significant that this precipitation is coincident with the sudden bend in the dissociation curve of the 1 : 24-acid.

We may describe the scope of the theoretical problem by saying that it must be possible to decide from a knowledge of the compound whether it will form phosphotungstates either (*a*) with dissociated basic groups (example creatinine), or (*b*) with dissociated groups which precipitate only when the

influence of other groups ceases to operate (example histidine), or (*c*) complexes with undissociated basic groups, insoluble in alkaline solutions upon the lines of guanidine. A complete solution should enable us to predict much about the constitution of an unknown base precipitable by phosphotungstate from its behaviour to c_H, and conversely the most likely method of selecting a base from some biochemical mixture. Fortunately the complication introduced by guanidine is not important unless precipitation starts at an alkaline p_H. It is

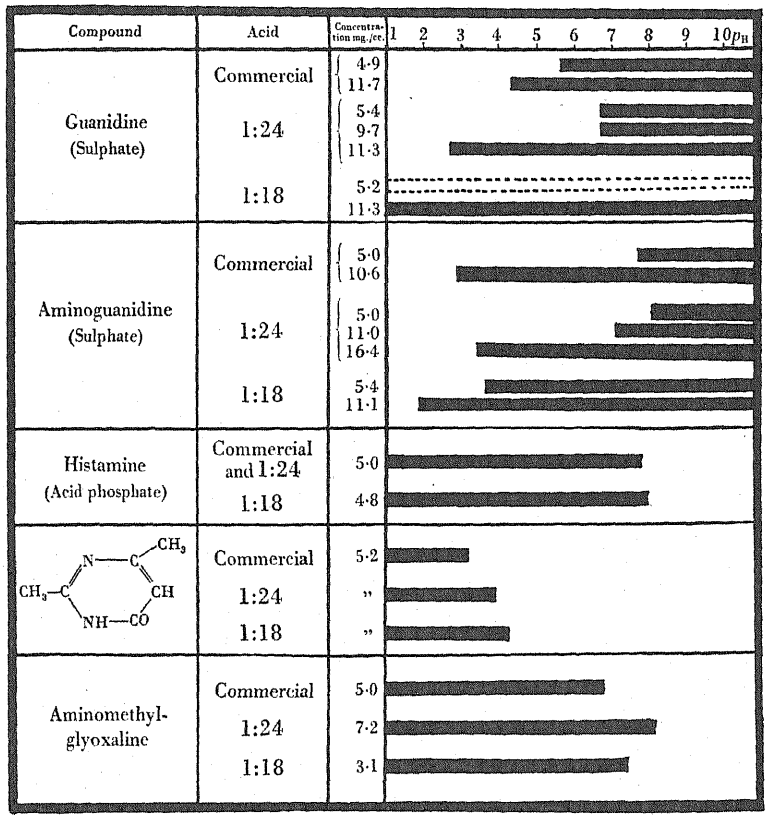


Fig. 5. Zones of precipitation (p_H), marked as black lines, for various compounds with three different phosphotungstic acids.

likely that substances attached to guanidine-like compounds are difficult to free from phosphotungstate with baryta. This may explain the losses sometimes found at a phosphotungstic stage. For instance Kinnersley and Peters [1925] found it to be quite impossible to recover vitamin B_1 from phosphotungstic acid precipitates at an early stage in their work.

The precipitates at acid p_H will usually dissolve at an alkaline p_H, upon addition of NaOH at a point some 2·0 p_H units more alkaline than the initial precipitation point, but there is a tendency to decomposition.

Some practical issues.

The applications of this work to the means of (1) characterising and (2) separating bases may be shortly considered in the light of these facts.

(1) It must now be realised that the original method of precipitating in the presence of minimum amounts of phosphotungstate must be rigorously watched and estimates of p_H of initial precipitation made at concentrations above the indeterminate zone. Solubility of the phosphotungstate matters much less in the case of substances like histidine or those like vitamin B_1 in which the vitamin compound is very insoluble. The latter precipitates at a dilution of less than 1 : 100,000 at p_H 5·0 [Kinnersley, O'Brien and Peters, 1932]. Presumably here the vitamin must precipitate very close to the beginning of its dissociation curve. (2) Several points are important in dealing with separations. The method used by Kinnersley and Peters [1930] for the separation of vitamin B_1, where the phosphotungstate is kept in minimum concentration, gives good results and has the great practical advantage that the amounts of phosphotungstate used are much smaller than the amounts needed to precipitate all the bases. This saves an expensive reagent. Their method, determined empirically, of working at different dilutions and gradually increasing the strength in successive fractionations is theoretically sound. Provided that a base precipitates between p_H 2·0 and 7·0, it should be possible to separate it by the use of suitable values of p_H and varying precipitations at various dilutions. These methods might be combined with fractionation in very acid solution. It is of course essential that the phosphotungstate should be very insoluble.

Precipitates at p_H 2–7 will give Na as well as organic bases upon decomposition. If therefore complete freedom from base is required, such precipitates should be treated with $N/10$ acid to free them from Na before decomposition with baryta. It is interesting to note that these principles of separation have been successfully applied at one stage in the separation of vitamin B_4 [Barnes, O'Brien and Reader, 1932], the vitamin appearing in the precipitate more acid than p_H 3·0.

Summary.

The precipitation of creatinine and of histidine HCl by pure phosphotungstic acids has been studied in detail and some data about the precipitation of certain other bases have been determined. The dissociation curves of the acids have also been investigated, and the data considered to some extent theoretically, but especially in their practical bearing upon newer methods of separating bases by the sodium phosphotungstate technique.

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